STUDIES ON THE VIRUS OF EPIDEMIC PAROTITIS (MUMPS) AND THE IMMUNITY REACTIONS TO IT IN MAN AND EXPERIMENTAL ANIMALS

BY

B. GHOSH RAY,

M.B., B.S. (CALCUTTA UNIVERSITY)

THESIS SUBMITTED TO THE UNIVERSITY OF EDINBURGH FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE FACULTY OF MEDICINE.

FEBRUARY, 1953.

CONTENTS

(Volume I)

(Volume II)

Section/

CHAPTER I.

Introduction and Review of the Literature

(SECTIONS A, B and C)

INTRODUCTION

No clinician has any difficulty in making a diagnosis in a typical case of mumps. The gross swelling of the parotid glands and the pain that accompanies eating and swallowing are so characteristic of the disease that laboratory aids to diagnosis are quite unnecessary.

Mumps, however, is a generalised infection and the parotid glands are not always affected; other organs may be involved and the complications of benign meningo-encephalitis, orchitis, pancreatitis and oophoritis are all well recognised. In the past these complications were only recognised as manifestations of a generalised mumps virus infection when there was clear evidence of parotitis. During the last ten years new techniques of the propagation of the virus in the chick embryo have made it possible to evolve serological and cultural methods of diagnosis and to show that occasionally individuals may suffer from illnesses due to this virus without any involvement of the parotid glands whatever.

A benign "aseptic" meningitis is perhaps the commonest form of this type of infection and is accompanied by the appearance of specific antibodies to the mumps virus in the blood stream. The differential diagnosis on clinical grounds lies between non-paralytic poliomyelitis, benign lymphocytic meningitis and other forms of "aseptic" meningitis. There/

There is, therefore, a considerable need for a diagnostic serological test in complicated cases of mumps. Several tests have in fact been evolved and their relative values have been outlined and analysed by different workers. However, the conclusions reached have often been contradictory, so that opinion is still divided on the practical utility of the tests especially for diagnosis in the early stages of the disease.

It has been known for a long time that an attack of mumps ensures a life-long immunity analogous to that after infection with the viruses of small pox or of yellow fever. Routine use of the serological tests has revealed that persons exposed to the mumps virus may suffer from 'subclinical' infection only and become resistant to further exposures to the virus. Both these groups of cases demonstrate a rise of the specific antibody content in the blood. But what determines the immunity state and differentiates it from susceptibility is not yet known.

The mumps virus is generally regarded as homogeneous and in the literature there is almost complete unanimity that there is little difference between the strains so far examined. This finding is remarkable and is in contrast with other haemagglutinating viruses; the influenza and the Newcastle disease viruses which belong to this group, are extremely/

extremely plastic and antigenically variable. Recent serological work has revealed a relationship between the mumps and the Newcastle disease viruses which may be explained by the sharing of a common antigen or antigens. It is not however known whether all the mumps strains contain this antigen.

Strains of the mumps virus when freshly isolated in Edinburgh were found to show at least minor differences of antigenic structures and in one of these strains considerable difference of habit of growth in eggs was noted. The fact that strain differences, however small, do occur is of importance when interpreting serological findings or when preparing diagnostic antigens. Moreover, in the latter case, the use of more than one strain or a 'local' strain is known to improve the accuracy of the serological reactions.

As an essential preliminary, a review of the literature as well as the recent serological observations on the immunity reactions in mumps are presented in this thesis. References relevant to the methods used in the experiments are reviewed at the beginning of each chapter. In addition to the review, an account is given of detailed studies made on the nature of the virus and on its modes and rates of reproduction in embryonated eggs. Serological methods, including those of complement fixation tests, have been adopted and extensively used/

used to investigate the phases of the virus growth and the appearance and fluctuations of antibodies in man and in small laboratory animals. The pathological effects that the virus produces in some of these animals have been studied.

The incidence of antibodies to the Newcastle disease virus in sera of individuals during an attack of mumps and after the recovery from infection was also investigated with a view to deciding whether the known antigenic relationship between the two viruses was constant.

A separate section has been included on the electron microscopy of different strains of the mumps virus. A short review of the principle and the application of this instrument in the study of the viruses, the hazards of the preparation of specimens, and the interpretation of the findings has also been outlined.

GENERAL REVIEW.

Mumps as a clinical entity has been recognised all over the world for centuries. Owing to the essential involvement of the parotid glands many earlier authors have described it as 'epidemic parotitis'. Various colloquial names like Oreillon (in French), Ziegenpeter or Banerwetzel (in Germany) have also been given to it.

The malady has been reported as early as the fifth century B.C. by Hippocrates, from an observation of its epidemic occurrence in the island of Thasos (Vaughan, 1922). He described the disease as a mild epidemic malady characterised by swellings near the ear which subside without suppuration and occasionally accompanied by painful enlargement of the testes. In these essentials mumps has not changed through the ages. In later times, Hamilton (1790) in Scotland was possibly the first to make a detailed study of the clinical features of the disease. He stressed the more frequent involvement of the testes and recognised the possibility of testicular atrophy as a sequela. Hamilton's observations also included a reference to occasional involvement of the nervous system in some cases and this has subsequently been emphasised by other observers. Thereafter it was realised that many other organs of the body, notably pancreas and ovary, might also be involved either during or after the parotitis. (Rolleston, 1932; Wesselhoeft, 1940).

Various /

Various authors have from time to time stressed the importance of particular clinical features or involvement of different organs from numerous observations in epidemics or in localised outbreaks. Only those that have a direct bearing in understanding the parasite or the reactions on the part of the host need be considered here.

Until the beginning of 18th century very little was known of the epidemic manifestations of the disease. Wide outbreaks were noted in Italy (in 1753, 1782) in Sweden (1851, 1856), in Denmark (1854, 1855) and many other parts of the world and the epidemic character of the disease was thus recognised (Rosenau, 1936). Further instances of prevalence of mumps within confined limits like prison camps, orphanages, convalescent homes, army barracks, schools, etc., led prominent workers to a belief in the contagious nature of the disease as was first pointed out by Protalango (Vaughan, 1922). On epidemiological observations, the saliva discharged from infected patients was attributed as the factor responsible for the spread of the disease.

Attempts to define and isolate the causative agent of mumps were begun in 1881 by Capitan and Charrin. Since then various microorganisms have been held as the responsible agent. It was only as late as 1934, that Johnson and Goodpasture, confirmed the /

the viral etiology of the disease, suggested earlier by Granata in 1908.

Capitan and Charrin (1881) found coccal and bacillary organisms in blood and saliva of persons suffering from mumps. These disappeared with the subsidence of the disease, after attaining a maximum when the disease itself was at its height. They grew easily in artificial media; but when injected in dogs, rabbits and guinea-pigs by various routes there was no obvious effect. Similar studies were continued by Laveran and Catrin (1893), McCray and Walsh (1896), Craig (1897), Haden (1919). All these authors identified one or other form of micro-organisms which however never fulfilled Koch's postulates.

Kermorgant (1925a,b) advanced a spirochaetal etiology of mumps. He isolated a spirochaete in association with a gram-negative bacillus from the mouth of mumps patients. Agglutinins and lysins were described in the convalescent sera of these patients which specifically reacted with these spirochaetes. He reproduced parotitis in monkeys by injecting both the organisms either into the duct or in the parenchyma of the gland directly. In rabbits, the intratesticular inoculation resulted in orchitis, which Kermorgant claimed was transferable. He was however/

however unable to demonstrate the spirochaete in the infected tissues microscopically. His results are not generally accepted by subsequent workers (Johnson and Goodpasture, 1934; Findlay and Clarke, 1934) who failed to detect any spirochete from parotid secretions.

While ordinary bacteriological cultural methods were proving a failure for the isolation of the causal agent Granata (1908 a,b; and see Kolle and Wassermann, 1913) struck a new note in these investigations. He thought a filtrable agent might be responsible. Using Berkefeld filtrates of saliva from infected patients, he produced non-suppurative parotitis in rabbits by direct inoculation in the substance of the gland. Intravenous injection of similar material only resulted in a pyrexia of 3 days duration. Comparable material from normal persons had no such effect. The non-suppurative parotitis resulting from the filter-passing material thus strongly suggested a viral etiology of the disease, although its relationship to human mumps was by no means established.

In the same year, Nicolle and Conseille (1913) used the gland puncture material from a child with parotitis for intraperitoneal inoculation in Bonnet monkeys. Some of them showed pyrexia of 4 to 6 days duration. In one of these there was also a left-sided/

left-sided parotitis. The authors contended that the pyrexia was specific. The monkey which developed parotitis became immune to a subsequent inoculation.

Gordon (1914) injected similar bacteria-free filtrates of saliva (saline gargle) into the brains of monkeys (M. rhesus and M. cynomolgus). The result was rather disappointing since all of them died. Some of them, however, showed symptoms of meningitis before death. This was of lymphocytic in nature and free from visible or cultivable bacteria. Microscopically, extensive degenerative changes in the cortical neurones and the anterior horn cells were observed. The only interesting feature of this series of experiments was the development of an interstitial parotitis in the particular monkey which survived longest. Simultaneous intravenous and intraperitoneal injections of the same material in a monkey resulted in bilateral parotitis after 11 days, with accompanying symptoms of meningeal irritation. However attempts at 'transference' experiments were unsuccessful. Besides the above, Gordon also showed that the causative agent was apparently heat labile; heating the infected filtrate at 55°C for one hour removed the power to produce meningitis.

Experiments of Wollstein (1916, 1918, 1921),

on/

on the above lines were more extensive. Similar filtrates of saliva from children in the acute stage of mumps were injected into the parotid, testis or the brain of cats producing inflammation in each of these organs after varying intervals of 5 to 9 days. There was pyrexia either preceding or accompanying the inflammatory conditions. She showed for the first time that the disease could be transferred in series. The disease was at its maximum between fourteen to seventeen days and the best results were obtained when serial transfers from affected glands were made at this time. Such transfers were also possible with infected brains of these cats. Woolstein further observed that by the sixth day of the disease, the infected saliva from patients was far less active to produce these inflammatory reactions. By the ninth day such activity was completely lost. Immune serum from patients had inhibitory action on infected gland emulsion. There was a diminished reaction in cats whenever the immune serum and the infected saliva were incubated together for sometime previous to inoculation. The organism in gland suspensions was however quite resistant in the presence of 50% glycerine for at least 4 months.

This review of the earlier literature shows the/

the causative organism of mumps variously designated by different investigators as a bacterium, a spirochaete or a virus. Wollstein's results were more convincing, although the relation to the human disease was not confirmed.

Johnson and Goodpasture (1934) not only completed this link of etiological relationship, but their experiments and observations were highly informative. They gave a broad picture of the nature of the virus and its pathogenic effect on the susceptible host. Their subsequent experiments (1936) served to explain certain aspects of immunity in mumps and the cytotropism of the virus.

Like other workers Johnson and Goodpasture used in their experiments saliva from infected patients. They did not however use filtrates for the inoculation. Injections given through the Stensen's duct in monkeys (M.rhesus) always produced typical parotitis. Parotitis followed a period of pyrexia which remained for 1 to 3 days. Swelling and tenderness of the glands subsided by two to four days. In fact such infected monkeys for all practical purposes presented the feature of clinical mumps in man. A serial transmission of the virus in monkeys by injecting the infected parotid gland emulsion was maintained for at least eleven passages./

In each of these passages an identical picture of parotitis was elicited. Proof of the etiological relationship to human mumps was finally established when the passage material (11th) was injected into the oral cavity of a group of susceptible children and produced typical parotitis in six out of thirteen cases. There was an incubation period varying from eighteen to thirty-three days. (Johnson and Goodpasture, 1935). None of a group of four children who suffered from mumps previously showed any sign of parotitis.

A number of other significant findings were also evident from their experiments . The serial passage in monkeys seemed to enhance the virulence for these animals. Later passages showed more nervous manifestations accompanying parotitis, than the earlier ones. On the other hand, the induced disease in children was milder than is usually seen during epidemics.

The animals which recovered from the experimental infection resisted further inoculation by the same route (parotid ducts). The surest way to bring about immunity in these animals was by infecting them through the parotid glands. Even unilateral infection of the gland was effective in preventing/

preventing the infection of the contralateral gland.

A clinical infection in monkeys can only be elicited by direct inoculation into the parotid duct. Oral spray of infected material is never followed by parotitis, although a state of subclinical infection results. This is also followed by development of immunity, which however is not so effective as that resulting after an attack of parotitis. A similar subclinical infection with development of partial immunity is also produced when a normal monkey is placed in contact with an infected one.

Intracerebral inoculation is followed quite regularly by the development of immunity. A subsequent 'challenge' dose of the virus given through the parotid duct fails to elicit parotitis. Inoculations through other routes like the intravenous, subcutaneous or intramuscular never give rise to parotitis and the resulting immunity if any is inconsistent.

Johnson and his co-workers also studied the histological changes in the parotid glands and the brain following inoculations. They described focal degeneration and necrosis of acinar cells of the parotid gland. Intracyto plasmic basophilic inclusion bodies were also detected and these authors claimed them/

to be specific. On the basis of the additional histological evidence both in the brain and the nerves leading to the parotid glands Johnson and Goodpasture (1936) favoured the view that a neural spread of the virus from the central nervous system to the glands is possible.

Their observations are also at variance with those of Gordon (1914) and Wollstein (1921). The presence of the virus in the brain and spinal cord of human cases of mumps is now beyond doubt since it has been isolated from the cerebro-spinal fluid in cases of meningo-encephalomyelitis (Swan and Mawson 1943 ; Henle and McDougall 1947). Philibert (1932) on the basis of clinical studies and on the available evidence during his time advanced the theory that mumps infection is primarily an invasion of the central nervous system — other organs being affected secondarily.

Findlay and Clarke (1934) confirmed in general Johnson and Goodpasture's work. They passaged the virus through monkeys for six generations. Intratesticular injection caused orchitis. They made a closer study of the histopathology of orchitis. Other laboratory animals like mice, rats and guinea pigs were also inoculated intracerebrally with the infected parotid gland suspension of monkeys. However/

However, they failed to show any sign of ill health.

Levaditi and his co-workers (1935a, b, c) challenged the specificity of parotitis in monkeys as shown by Johnson and Goodpasture (1934). They argued that other viruses like that of Nicolas-Favre disease (Syn: Lymphogranuloma venereum), when inoculated into the parotid gland of monkeys produce an inflammatory reaction not differing materially from that obtained with the saliva of individuals suffering from mumps.

Bloch (1937) re-examined the specificity of lesions in the parotids of monkeys under carefully controlled conditions. He concluded that although cytoplasmic inclusion bodies can be found in 50% of cases of mumps infection in monkeys, bodies of similar nature, except being numerically less, can be found in some of the controls; for this reason the inclusions as described by Johnson and Goodpasture could not be described as specific. The only observable fundamental lesion in the infected gland was focal acinar necrosis.

The results so far obtained, clearly demonstrated that the monkeys (M.rhesus and M.cynomolgus) were quite susceptible to the virus. They could be used for the isolation of virus. Enders and Cohen in 1942 showed that the parotid gland suspension from monkeys/

monkeys with parotitis could be used as a suitable antigen for a complement fixation test to demonstrate specific antibody developed in sera of mumps patients. From then onwards monkeys were used extensively for all work on experimental epidemiology.

In the meantime Goodpasture and his collaborators (1938) made use of fertile hen or duck's eggs in growing some other viruses and bacteria. Such a method of cultivation was utilised for investigating various problems in bacteriology and serology with profitable results. Habel (1945) applying those methods succeeded in cultivating the virus of mumps in embryonated eggs. He used the infected parotids of monkeysasa source of virus. Presence of growth was detected by the application of the methods of Enders and Cohen (1942) in using convalescent human or monkey serum to titrate the complement-fixing antigen i.e. the virus content in infected fluids. The virus grew in all the three embryonic fluids, namely, the allantoic, amniotic and the yolk. After growth, the virus was identified again by intraductal inoculation in fresh monkeys who developed pyrexia and typical parotitis. Habel thus showed that the embryonic fluids could be used as a ready source of virus either for the study of the nature of the organism itself or as antigens

for/

for complement fixation tests with patient's serum. He also utilised such fluid as antigens for eliciting skin tests in a study on the immunity of the disease.

Levens and Enders (1945) cultivated the virus in eggs on identical lines; they however extended the observations of Hirst (1941), and McClelland and Hare (1941) to show that the mumps virus also possessed a haemagglutinating factor analogous to that of influenza virus.

Beveridge and co-workers (1946) applied the egg-cultivation methods to isolate the mumps virus from saliva of infected patients. Sulphonamide solution was injected in eggs to prevent bacterial contamination. In four out of twelve cases virus was isolated by yolk sac inoculations. Amniotic inoculations were also found equally effective in the isolation of virus from human saliva. The complement fixation test with known immune sera was used for detecting the presence of growth of the virus and the results were checked by testing for the presence of haemagglutinin as well. It was also found that the haemagglutinating property may be entirely absent in the first passage and that a few subsequent passages are needed before it can be detected.

For the comparative ease with which the virus can be cultivated in egg-fluids and the growth detected by its haemagglutination property, the egg cultivation/

cultivation method was soon applied by many investigators in their respective domain of research. Rapid strides have been made in the study of the nature and the biological behaviour of the virus. These have resulted in the development of new serological techniques. At present it is possible to diagnose the infection in the laboratory on cultural and serological grounds and indications may be obtained on the immunity or susceptibility of an individual. The observations made by different authors and the results of their investigations cognate to the subject of the present research have been briefly outlined in the following pages of the review.

IMMUNITY IN MUMPS AND THE SEROLOGICAL REACTIONS.

It has been commonly observed that the recovery from infection with a virus, in most cases leaves varying degrees of specific resistance to re-infection. In some instances this immunity is apparently solid, lasting and effective. This observation has in the past led to an assumption that the nature and the underlying mechanism in the production or the mode of action of the antiviral antibody, is different to that resulting from bacterial infection.

Such a generalisation is not however warranted by facts. According to Rivers (1948) "The basic principles of immunology and serology are operative in all fields of biology. Consequently, one should not be surprised to find familiar immunologic and serologic phenomenon associated with viral and rickettsial diseases as well as with other infectious diseases". The same view has been expressed in the reviews on the subject by other authors (Bedson 1937a; Burnet, Keogh and Lush, 1937; Andrewes, 1939). Wilson and Miles (1946) in a summary on the subject state that "the difference is due rather to the general limitations imposed on the virus by its habit of intracellular parasitism than to any special reaction on the part of the host".

Thus,/

Thus, three kinds of immunity, namely natural, actively acquired and passively acquired, are also met with and have their bearing on the phenomenon of resistance or susceptibility of the host to the viruses.

The natural immunity is not infrequently dependent upon several factors such as age, species, climatic conditions, etc. Whereas these are true for most of the viral and other infectious diseases, two more factors have been recognised in the former group of diseases that greatly modify the susceptibility of the host. The fact that the tissue or cellular affinity of a particular virus influences the chances of infection is well known and needs no further elaboration. The second factor has been recognised recently and is described under the term 'interference phenomenon'. The exact part it plays in immunity to virus infections is not very clear. But the experimental evidences have created a great theoretical and practical importance. The probable explanation for the phenomenon and of its role in the immunity has been discussed recently by Henle (1949a, 1950).

Active immunity in virus diseases always results from previous exposures to the virus. The immunity may be solid and durable in some diseases while/

while in others it may be quite short-lived. It is characterised by the appearance of specific humoral anti-bodies. The neutralising and complement-fixing anti-bodies, agglutinins and precipitins have all been demonstrated. It is believed that the neutralising anti-bodies in certain instances are different from the others and are responsible for protection (Rivers 1948). However, it has also been found that some experimental animals recovering from a virus infection do not show the neutralising anti-bodies in their blood although they are resistant to re-infection. This state of immunity has been wholely or in great extent attributed to some special and specific changes in the tissue cells. Levaditi (1926,1946) has been an exponent of the importance of the cellular or tissue immunity in virus diseases. The work of the last three decades has however provided overwhelming evidence that the humoral factor is all important in acquired immunity to virus infections and the role of pure tissue immunity, if any, is only secondary (Bedson 1950).

The durable immunity resulting from an infection of mumps, as indicated by the very low rate of "second attacks" has always surprised the investigators of the past. There has been much speculation on the mechanism which confers this lasting immunity. It has been suggested from animal experimentation (Olitsky and Long 1929; Bedson 1938; Olitsky and Casals 1945) that even after clinical recovery the infection does persist in/

in a refractory state and the causative virus may still be recovered. It has also been argued that a similar situation exists in human convalescents who are regarded as 'carriers'. In them the persistence of the virus serves as a repeated antigenic stimulus whereby the antibody persists in effective concentration even in the absence of re-infection. On epidemiological evidence it has also been suggested that sub-clinical infections may account for the antigenic stimuli. The epidemiologists have reckoned that there is a high incidence of mumps in children. Although most of the children are usually affected, quite frequently some may escape an attack in institutional or household outbreaks, even in spite of close contacts (Gordon and Heeren, 1940; G. Henle et al. 1948a, b).

That immunity of considerable degree is present in the general adult population has also been observed. Adults who live regularly in urban areas usually escape infection. Not in all cases do they suffer from mumps in their childhood. An analysis has revealed that only about 60% of the adult population in the United States of America has experienced a recognisable attack of the disease (Maris et al.1946). On these grounds it has been suggested that the lower incidence of mumps might result from specific immunity induced by subclinical infection. It is also known that in the absence of exposure to the virus there is practically very little development of resistance to the disease. Thus, there is a high incidence of mumps even in adults when they migrate from rural area../

area. Frequent outbreaks are noted in army barracks and training centres, in schools or jails or in closed communities such as institutions.

In order to seek an explanation of these epidemiological findings of mumps and for the formulation of public health measures, investigations have mainly been pursued on the following lines:-

(1) In devloping suitable techniques for the diagnosis of the disease, particularly in doubtful cases of parotitis and in cases which present symptoms other than parotitis.

(2) Detection by serological or other means of cases of subclinical infection and 'carrier' states, if any.

(3) Determination of the criteria of immunity after infection and the susceptibility or resistance in persons who have not suffered clinically.

(4) To find out means of conferring effective active immunity upon susceptible individuals.

(5) Development of methods to assay the protective properties of convalescent sera for prophylactic or therapeutic use.

In recent years the earliest evidence of a neutralising anti-body against mumps was demonstrated by Johnson and Goodpasture (1934) in their experiments with the monkeys. They found that one attack of parotitis made these animals immune to subsequent challenge with a larger dose of virus. The sera from such animals, as well as the mumps convalescent human sera, exhibited a certain degree of inhibitory effect on the virus. The authors could not determine the quantitative potency of this inhibitory effect/

effect because of certain limitations; these, they said, were the difficulty in determining a minimum infecting dose and the exceptionally large amount of virus required to infect the animals.

However, a measure of antibody content was soon made possible by Enders and Cohen, (1942). Using the infected monkey (rhesus) parotid gland suspension as antigen they evolved a complement fixation test to demonstrate that the antibodies develop regularly both in artificially infected monkeys and in natural infections of human beings. As a result of further extensive studies on various aspects of immunity in mumps, Enders and his co-workers (Enders, Kane, Cohen & Levens, 1945; Enders Cohen and Kane 1945; Kane and Enders, 1945) found comparable results with this complement fixation test both in human sera and monkey sera.

In monkeys the complement fixing (c.f.) antibody appears in the blood at about 8th day after intra-ductal inoculation of the virus and reaches a maximum concentration between the 13th to 15th day, i.e. even before the onset of parotitis which usually is manifested on the 18th or 20th day after injection. A high complement-fixing antibody level is maintained for 1 to 2 months and there is a gradual fall afterwards. It has even been detected after 21 months at a low titre. A similar rise in antibody, although not to a high titre, has also been observed in monkeys subjected to subclinical infection and in those undergoing inapparent infection from close contact.

Analysing/

Analysing 48 typical cases of epidemic parotitis, these authors concluded that the complement fixing antibody developed de novo or increased in concentration during convalescence. Thirty four of these cases revealed no antibody either before the disease or during its early phase. In the rest, the initial or acute titres were all quite low while a significant increase in antibody was subsequently demonstrated. The antibody was also found to be specific.

The complement fixing antibody appears very rapidly with the onset of symptoms, even within 1 or 2 days. But usually it is demonstrable between the 5th to 7th day after onset increasing greatly in amount during the 2nd. week. By the 14th day, practically all convalescent sera show high titres of the antibody. The level of antibody starts falling after 6 weeks to 8 weeks and by six months or one year, reaches a very low level. Such a persistence of antibody in a low level, although not universal, has also been detected by other workers (Jordan and Feller, 1950).

Habel's (1945) demonstration of the growth of the mumps virus in the different fluids of embryonated eggs revealed the suitability of these infected fluids as a complement fixing antigen for which purpose they have now been universally adopted and have supplanted altogether the monkey parotid gland emulsion antigen. Complement fixing antigens variously prepared by different investigators from

different/

different components of infected chick embryo have been used. Recently, they have been prepared from the infected allantoic fluid and from the chorioallantoic membrane itself.

The nature of the complement fixing antigen, reacting specifically with the antibody, was identified (Lind 1948) as part of the virus itself. Henle, Henle and Harris (1947) showed that there exist at least two serologically distinct antigens; one which is linked with the virus particle and found predominantly in the infected allantoic fluid is called viral or 'V'-antigen, while the other smaller in size and chiefly present in infected allantoic membrane is called soluble or 'S'-antigen.

An analysis of more than 2,000 sera from several hundreds of cases of epidemic and experimental mumps in children (Henle, Henle, Wendell and Rosenberg, 1948) has revealed that the serologic response to two different complement-fixing antigens usually follows a distinct pattern. Antibodies against the soluble or S-antigen, as a rule appear to reach high titres before the antibodies against the virus-bound or V-antigen can be detected at significant levels. Frequently the anti-'S' antibody attains distinct levels at the time signs and symptoms of the disease appear. In some other cases, the disease becomes overt, before complement fixing antibodies can be demonstrated. Exceptionally, the anti-V antibodies appear simultaneously with or even prior to anti-S. In early convalescence, i.e. about 6 to 8 days after the onset

of/

of mumps, the titre of anti-V antibody usually exceeds that of anti-S. Subsequently the antibody-titres against both V and S decrease, anti-S at a faster rate, as a rule, than anti-V. Thus, several years after the infection, anti-V may still be measurable without any evidence of the anti-S antibody. It has however been observed that both antibodies may disappear from serum completely. Both the V and S antigens are therefore helpful for diagnostic procedures. The early appearance of anti-S antibody when the anti-V antibody is still low, is frequently seen in cases of mumps-encephalitis without parotitis (Henle and Henle 1949a). This general pattern of the serologic reactivity is also seen in subclinical infection of mumps with a high degree of regularity (Henle et al. 1948a, b).

After comparing the data published by Enders and his co-workers with their own, Henle, Henle and Harris (1948a) suggest that the antigen contained in suspensions of infected parotid glands of the monkey is dominantly of the S-type. This assumption has been based on two facts: firstly high antibody levels against monkey parotid antigen are usually found on the first few days of the disease, and secondly, the incomplete fixation of complement and the marked zoning, which have been observed in occasional sera in the acute phase of disease, when monkey parotid antigen was used, is found also to occur when employing the 'S' antigen of chick embryo, though not with the V-antigen.

The/

The value of complement fixation tests employing soluble or 'S'-antigen and viral or 'V'-antigen has been confirmed by Rice and Walker (1948) and Oldfelt (1949). Rice (1949) has even evolved a test for quantitative determination of the antibody content against the two antigenic components. Other workers have extensively used either both the complement fixing antigens (Aikawa and Meiklejohn 1949); (Florman and Kutch 1949); (Feller and Jordan 1950) or only the viral antigen (Beveridge and Lind 1946); (Robbins, Kilham, Levens and Enders 1949); (Lundback 1949) for evaluating the different serological tests for diagnosis of mumps on comparative basis.

Levens and Enders (1945) and later Burnet and his co-workers (Burnet, 1946; Beveridge and Lind 1946) observed that the mumps virus grown in embryonated eggs will agglutinate fowl and human group 'O' erythrocytes in the same manner as the influenza virus. Following the methods of Hirst and Pickels (1942) and Salk (1944) for demonstrating haemagglutination-inhibiting antibody against influenza Levens and Enders developed a similar test to detect the haemagglutinating antibody against the mumps virus. They found that a specific antibody develops in the serum after mumps infection which will inhibit the haemagglutination by the mumps virus. The antibody is also detected in convalescent sera of the monkeys after intraparotid inoculations. The simplicity and the ease with which this test is performed has led/

led numerous investigators to apply it in routine diagnosis of mumps (Beveridge and Lind,1947; Florman and Kutch,1949; Aikawa and Meiklejohn,1949 etc.) Its applicability in diagnosis has been doubted by Lundbäck (1949) but the findings of other workers notably Robbins, Kilham, Levens and Enders (1949), Aikawa and Meiklejohn (1949) have shown its usefulness in diagnostic work.

In established cases of mumps, a high inhibitory titre is almost always obtained in the serum. The antibody appears within the first few days (even as early as three days) after the onset. Then there is a very rapid rise and at the end of two weeks the highest level is reached. At such a time there is on an average a 4 to 8 fold rise from the original titre. Even a 32 or 64 fold rise is not uncommon (Aikawa and Meiklejohn 1949). The specificity of the test has been demonstrated by Enders, Kane, Cohen and Levens (1945) and confirmed by subsequent workers (Aikawa and Meiklejohn 1949; Florman and Kutch, 1949; Robbins, Kilham, Levens and Enders, 1949; Feller and Jordan, 1950).

Non-specific inhibition of the haemagglutination by mumps virus has been found on many occasions. A comparative high level of this inhibitory titre has also been demonstrated in apparently healthy individuals and in some cases of infection even in the early stage of disease. The use of different batches of fowl cells for the test (Anderson, Burnet and Stone 1946; Lundbäck 1949) or variation in the amount and strain of/

of the virus (Beveridge and Lind,1946) cause great differences in titre in these sera. Even repeating the test on different days a 2-fold or more difference in titre can be detected (Lundbäck 1949); for these reasons the test is sometimes regarded as unreliable, particularly if a single sample of serum is examined.

Robbins, Kilham, Levens and Enders (1949) have critically evaluated the test. In their technique such variations in titre are reduced to a minimum and in no case has a non-specific inhibitory titre of more than 1/64 been encountered either in normal or acute phase sera. According to them, as well as other authors (Aikawa and Meiklejohn,1949; Feller and Jordan,1950), for diagnostic work paired samples of sera should always be examined and a four-fold or more increase in titre during the course of disease, may be interpreted as evidence of infection.

Burnet (1946) has shown that human Group-O cells treated with mumps virus are agglutinated. Subsequently when the virus is allowed to elute, these cells are not susceptible to agglutination by the same virus. Instead they are specifically agglutinated in presence of immune serum from mumps cases. He found marked difference in titre between the normal sera and immune serum. This treated-cell agglutination test (Burnet test) has also been utilised for detecting a rise of antibody in man (Beveridge and Lind,1947; Aikawa and Meiklejohn,1949; Florman and Kutch,1949) and in experimental animals.

Higher/

Higher numerical values of the titres are reached during convalescence, obviously giving a wide range of the antibody titre and increasing the sensitivity of the test. Even a 100-fold rise in titre has been found (Florman and Kutch). The test however, has not received universal support. This is because of the extreme difficulty of preparing the modified red cells (Henle and Henle 1949a). The cell suspensions frequently become auto-agglutinable and with very high-titre sera a prozone is observed in the lower dilutions. At times it is also difficult to recognise the end point of agglutination.

Neutralising antibody:

As has been mentioned earlier, Johnson and Goodpasture (1935) detected an inhibitory power of monkey convalescent sera on the monkey parotid gland virus. They could not however, measure it. Enders and his co-workers (1945) experienced similar difficulties. Their experiments showed that the neutralising antibody is closely associated with the gamma-globulin content of the immune sera. A purified and concentrated gamma-globulin fraction prepared from convalescent mumps sera definitely inhibited the virus to a marked extent.

Hirst in 1942 demonstrated that the neutralizing power of convalescent sera from influenza patients could be titrated by an "in ovo" protection test. Habel (1945) employed similar methods in studies on immunity in mumps. He found distinct differences of the antibody content between convalescent sera and the sera of acute phase, or from normal persons. In many cases the neutralising antibody was demonstrable in those sera which contained also the complement fixing antibody; but no quantitative correlation between the two antibodies was evident.

Leymaster and Ward (1948) systematised the test/

test for quantitative titration of the antibody. They agreed with Habel's findings that definite difference in the antibody content was present between the immune and non-immune sera. A correlation between the positive titre of the test and positive history of previous mumps infection was also possible.

More recently Bashe et al.(1952) have communicated their attempt at standardisation of the test and their findings on the relative values of different serological tests. They noted that in early convalescence the neutralizing antibodies show a high rise in titre like the haemagglutination inhibiting and complement fixing antibodies. In some cases, however, one or the other tests gave negative results. The three methods in all cases did not also yield positive results. They found that sera from random individuals as a rule gave negative results where the history of mumps and the results of the complement fixation test were also negative.

In an attempt to systematise the neutralization test Leymaster and Ward (1948, 1949) observed that fresh unheated serum is essential for the test. They found that a heat-labile component of fresh serum potentiated the neutralising capacity of

of the immune serum; the potentiating effect was reduced or destroyed when the immune sera was left at room temperature for sometime or heated at a higher temperature. When heated serum (inactivated) was used for the test only a very low titre was obtained. Reactivation of the heated sera was possible by the addition of fresh normal serum. From the results of an elaborate experiment these authors suggested that the potentiating factor of fresh serum was identical with the complement. The presence of a similar heat-labile component in the sera of humans, guinea pigs and rabbits which can neutralise mumps virus in presence of calcium salts has also been recognised by Ginsberg and Horsfall(1949b). They, however, identified it as a protein distinct from complement.

Two or more of the serological tests mentioned above have been applied by various investigators in their attempts to evaluate the antibody response to mumps infection. The observations on individual tests have been already outlined briefly. It is appropriate at this stage to mention their relative values for diagnostic purposes.

On examining sera of monkeys infected with mumps, Enders, Levens, Stokes and Maris (1946) found that the complement fixing antibody can be detected in blood on the 12th day after infection and reaches the/

the maximum level at about the 14th day. The anti-haemagglutinin on the other hand does not appear until at least two days after it and attains maximum concentration about 6 days after the complement fixing antibody has reached its highest level. In man, the complement fixing antibody appears between 5 to 7 days after the onset of the disease. (Enders, 1946). In some cases it is not unusual to detect it earlier, even on 4th day (Aikawa and Meiklejohn, 1949), or on the day of onset (Florman and Kutch, 1949). The anti-haemagglutinin appears 2 to 9 days after the onset and rises to a maximum level more rapidly than the complement fixing antibody. The agglutinin for treated cells (Burnet cells) appears on the 10th to 15th day of the disease (Florman and Kutch, 1949).

There is a difference of opinion on the time of appearance of anti-V and anti-S antibodies. The contention of Henle and his co-workers have been challenged by all subsequent investigators. Thus, Aikawa and Meiklejohn (1949) have found at least a two-fold lower titre of the anti-S antibody when compared with the anti-V antibody, in 10 out of 11 cases. The higher level of anti-V antibody was consistently maintained in later stages of the disease. Florman and Kutch (1949) have made similar observations./

observations. They detected the anti-V antibody earlier than the anti-S antibody in most of their cases. In some the anti-S antibodies were even found after the appearance of the antihaemagglutinin. Feller and Jordan (1950) have observed that although the anti-S antibody appears in some cases earlier than anti-V, the results are most inconsistent; it may even fail to appear. In none of their cases was anti-S antibody detected where anti-V antibody was absent. They concluded that in the majority of cases anti-S antibody was of no value for a diagnosis in the early days of the disease.

In spite of such differences of opinion on the appearance of the two components of complement fixing antibody it is generally agreed that the complement fixation test is more reliable for routine diagnosis. It is more regularly demonstrated than the haemagglutination-inhibiting antibody during the early days of the disease. Further, the stability of antigens over long periods makes it a valuable diagnotic test. The test gives significant results frequently with a single serum taken during the first days of illness. This has a great advantage over the haemagglutination-inhibition and the Burnet test which almost always require the comparison of sera taken during the acute and convalescent stages of the disease.

Inhibition/

Inhibition of Mumps haemolysin:

Recently it has been shown by Morgan and his co-workers (Morgan et al., 1948; Chu and Morgan 1950 a,b) that the mumps virus grown in extra-embryonic fluids exhibits the power to haemolyse the erythrocytes from man and other species of animals. They showed this property (reviewed elsewhere) to be closely associated with the virus particle itself. In an attempt to detect the presence of any anti-haemolysin in the convalescent sera of mumps patients, they found that the haemolysis could be inhibited with immune sera even in high dilution. Normal monkey sera or sera from the acute stage of mumps infection had very little inhibitory power. The test has not been standardised so far for diagnostic purposes.

Cross serological reactivity of immune mumps sera and the New Castle disease virus (N.D.V.).

Since Burnet and his colleagues (Burnet, 1945; Burnet, McCrea and Stone, 1946) described the "red cell receptor gradient" of mumps, Newcastle disease and influenza group of viruses, it has been postulated that the N.D.V. and mumps viruses may have some antigenic relationship. Indeed, a serological relationship between the two viruses was observed later by Kilham and his co-workers (Kilham, Jungherr and Luginbuhl, 1949; Jungherr, Luginbuhl and Kilham, 1949). They found that when sera/

sera from 22 mumps convalescent patients were tested against N.D.V., 13 showed significant reactions with the neutralisation test and 11 with inhibition of haemagglutination. Out of 23 controls with undiagnosed diseases of central nervous system 3 gave responses by neutralisation but none by the anti-haemagglutinin test against N.D.V. They observed that the neutralizing factor of the mumps sera against the N.D.V. was heat-labile (at 60°C for 20 minutes) and the anti-haemagglutinating factor was relatively heat-stable. The results were somewhat anomalous with anti-N.D.V. immune sera; whereas the immune fowl and rabbit sera did not give cross-serological reactions with mumps virus, the immune guinea-pig sera with high neutralization indices against N.D.V. also neutralized considerable doses of mumps virus.

The observations of Kilham and his co-workers were further extended by Jordan and Feller (1950). They confirmed the previous finding of Leymaster and Ward (1949) that the use of unheated and fresh mumps convalescent sera was essential to demonstrate the true content of the neutralizing antibody against the mumps virus. As the Newcastle disease virus is neutralized non-specifically by heat-labile factors in fresh serum they suggested that the use of

unheated/

unheated immune mumps serum was not very helpful in the demonstration of a serological relationship. Employing complement-fixation tests they found that 12 out of 15 paired mumps convalescent sera showed 4-fold or greater rise in titre against N.D.V. 74 single sera containing mumps antibody were also tested and in 39% of these the anti-N.D.V. factor was detected. It was found also that 91% of the sera showing antibodies against the N.D.V. contained complement fixing mumps antibody.

The serological cross-reactions were less marked when tested by haemagglutination inhibition test. Out of 12 sera which showed the presence of anti-N.D.V. factor, only 7 showed significant rise in the haemagglutination inhibition titre. The specificity of these two serological reactions was indicated by the fact that no 4-fold rise of titre was seen with either the c.f. test or the H.I. test during examination of 49 and 55 normal sera respectively. It was, however found that the anti-N.D.V. factor may be present in some sera independent of the presence of mumps antibody.

Jordan and Feller further found in a limited number of cases that sera of patients convalescent from influenza and from cases of infectious/

infectious mononucleosis did not show any increase in c.f titre or anti-haemagglutination activity against N.D.V. From these results they suggested that the N.D.V. and mumps virus may have a common antigen (or antigens) or that there are two or more types of mumps virus some of which may not simulate the N.D.V. factor.

CHAPTER II

MATERIALS AND METHODS

The general procedures employed for the cultivation of the viruses in embryonated eggs and their identification, and the routine serological tests performed during the present investigation will be described in this chapter. Explanations on the choice of these methods and their standardisation have been outlined in the next chapter.

In addition to those to be described below, other methods were employed for individual experiments and departures were made from the general methods. These have been described in the appropriate chapters.

Supply and Management of Eggs:-

Fertile brown Leghorn eggs supplied by the Department of Genetics, University of Edinburgh, were used in the present study. All the developing egg-embryos for a given experiment were from the same batch of eggs received on a particular day in this laboratory.

The general management of the eggs before inoculation were according to the methods described by Beveridge and Burnet (1946). The eggs were mostly clean when supplied. They were, however, washed in weak soap solution, in running tap water, and then dried before incubation. All the eggs were incubated in presence of moisture at 37°C. for 7 days. Later, those to be inoculated with the mumps virus were transferred into another incubator running at 35°C.; these, after inoculation, were further incubated at the same temperature before harvesting the fluids or the tissues. Those inoculated with influenza B (Lee) virus were also maintained at this temperature after the inoculation. After inoculation with other viruses such as the Newcastle disease virus or influenza A (PR.8), the incubations were carried out only at 37°C.

Preparation of eggs, inoculations and harvesting of the extra-embryonic fluids and tissues.

Allantoic inoculation:

The egg was candled to determine the limits of the air space; over the eggshell a point was selected where the chorio-allantois was well developed but free from large vessels. A small area surrounding the point was painted with iodine solution and a groove measuring 1 m.m. x 3 m.m. was drilled through the shell without damaging the membrane underneath it. The groove was then sealed with a drop of hot melted paraffin.

Before inoculation, the paraffin was melted with a hot metal spatula. An appropriate dilution of the inoculum contained in a tuberculin syringe was injected through the fine bore of a hypodermic needle by piercing the paraffin barrier. The needle was inserted to a depth of less than half an inch at an acute angle to the plane of the shell and during injection was held almost parallel to it. The needle hole was sealed again with hot paraffin wax. The volume of the inoculation was usually 0.1 to 0.15 ml.

After the eggs had been incubated for varying lengths of time and at varying temperatures, they were chilled at 4°C for 2 to 16 hours to kill the embryos and faciliate collection of allantoic fluids without the presence of much blood in it. The egg

shell over the air space was painted with iodine, drilled around its entire circumference and removed. With a sterile forceps a rent was made in the shell membrane and the chorio-allantoic membrane taking care to avoid injury to large blood vessels. The allantoic fluid could now be pipetted off. A separate sterile glass capillary pipette was used to harvest the fluid from each egg.

To harvest the chorio-allantoic membrane (C.A. Membrane), the egg was freed of its contents by holding the air sac end downwards on a petridish. The membrane usually sticks to the shell membrane and after freeing the contents can be peeled off with a forceps. The membranes were collected separately into test tubes and pooled when required.

When allantoic fluid was collected from inoculated eggs it was usually tested for sterility on blood agar plates. Virus content was determined by the haemagglutination test for routine purposes. The fluids showing a positive reaction and found sterile on culture were pooled and preserved by methods described later.

Amniotic Inoculation:

The candling of the egg was done as before. Shell-surface near the position of the embryo was cleaned with a cotton-wool swab soaked in 75% alcohol.

An/

An oval area of the shell (5 m.m. x 10 m.m.) was drilled and lifted free from the shell-membrane with the sharp point of a needle. Over the centre of the air space another groove was cut sufficiently deep to perforate the shell membrane. A drop of sterile saline was placed on the exposed oval shell membrane which was then punctured with the curved tip of a sharp needle; especial care is exercised so that the C.A. membrane lying deep to the shell membrane is not pierced. A gentle suction through the hole at the air-sac end helped to separate these two membranes with the production of an air space. Within the limits of this air space another oval area (1 cm. x 2 cm.) surrounding the earlier one was drilled through the shell wall; the shell wall and the exposed shell membrane were then removed by cutting with a pair of scissors along the margin of the groove. The hole was now sealed with a piece of transparent adhesive tape ('Scotch' tape) which could be easily stripped off before inoculation and replaced again.

The inoculation was made through a sharp-pointed hypodermic needle (gauge .27), fitted to a tuberculin syringe. A volume of air which just filled up the needle (about 0.05 to 0.1 ml.) was drawn in after the syringe had been charged with the inoculum. The needle was inserted through the chorio-allantoic/

chario-allantoic membrane at a place where it was comparatively avascular and overlaid the edge of the amniotic membrane. It was pushed towards the embryo when a portion of the amniotic membrane gathered up at its tip became stretched; with a light and jerky stab through the stretched-out membrane the amniotic cavity was reached and the volume of air contained in the body of the needle released. From the dull appearance of the air-bubble and its movement along with that of the embryo it is always possible to ascertain that the tip of the needle is actually inside the amniotic cavity. The inoculum, usually 0.1 to 0.125 ml. in volume was then injected, the needle withdrawn and the hole in the shell-wall sealed again with the 'Scotch' tape.

The method as described above causes a minimum injury to the C.A. membrane by avoiding any cut with scissors or the grasp with forceps as recommended by Beveridge and Burnet (1946). Besides, it has the additional advantage over other methods (Enders 1948 ; Hirst, 1942) in being an 'open' method where all manipulations could be clearly visualised.

For harvesting the amniotic fluid the eggs were chilled after the period of incubation. The 'Scotch' tape was peeled off and a wide area surrounding the hole painted with iodine solution. The/

The C.A. membrane overlying the embryo was torn with a forceps. Grasping the amniotic membrane with another forceps, the amniotic fluid was collected with sterile capillary glass pipettes. When required, the amniotic membrane was also collected by cutting it away from the "Stalk" with a pair of scissors. The fluids were tested for presence of virus and any bacterial contamination and then pooled as described earlier.

Harvest of extra-embryonic fluids and tissues for use as 'normal' controls.

Uninoculated eggs were incubated under identical conditions of time and temperature. They were chilled as before and the shell with the underlying shell membrane were removed by drilling from the air sac end. The allantoic fluid was removed and the contents were carefully drained into a sterile petridish taking care that the amniotic membrane did not rupture. The amniotic fluid was then removed with a capillary pipette. The membranes — amniotic and allantoic were collected as before. The components were pooled separately and preserved with a mixture of antibiotics.

Collection of erythrocytes from different hosts:

The red cells from man and other animals mentioned below have been used for haemagglutination tests/

tests or other experiments. They were obtained in different ways.

Human: Only group 'O' cells have been used. A regular supply of the same was obtained through the courtesy of the authorities of the blood bank in Royal Infirmary, Edinburgh.

Fowl: Adult cockerels were bled from their wing veins, the blood being collected in citrated saline to prevent clotting. The red cells from these birds had been tested before for their sensitivity to agglutination by the different strains of the mumps and the influenza viruses; only those that indicated high titres with most of the virus strains used were selected for bleeding. As far as possible, the cells from the same bird were used for any particular experiment. In no case the red cells from different birds were pooled for routine use.

Guinea pig, White Swiss mouse and Albino rat:

These animals were bled from the heart under light ether anaesthesia. The blood samples were collected in citrate saline;

on/

on some occasions only clotted blood could be obtained. In these cases, after separation of the sera, which were used for other experiments, the blood clots were crushed by shaking with glass beads. The resultant suspension was strained through thin layers of gauze or cotton wool in order to remove the clots.

Sheep: Obtained from a local slaughter house, the blood was treated with K-oxalate crystals to prevent clotting.

Rabbit: Citrated blood collected from the ear-veins.

Dog and Cat: Citrated blood collected by vein-puncture.

The red cells after collection were washed at least four to five times with normal saline by repeated sedimentation in an angle head centrifuge. They were packed by final centrifugation in ordinary round-bottom test tubes at 2,500 r.p.m. for 20 minutes. Requisite cell suspensions were prepared from the packed cells. Between experiments the cells were kept at 4°C, resuspended in sterile normal saline. They were re-washed before use. No cells older than four days have been used.

VIRUS:

The following viruses have been used during the present investigations.

Mumps:

(1) 'Enders' - Strain (E.M.A.41): A freeze dried specimen of the original 'Enders' strain had been obtained in this laboratory through the courtesy of the National Institute for Medical Research, Mill Hill, London. It was reported to have been through 41 egg passages before the drying. Since the receipt in this laboratory on 28.8.48 the dried specimen was stored at 4°C. It was reconstituted on 28.10.50 and passaged in the allantoic and amniotic cavity of 8 day-old eggs. Regular passages by the allantoic method have maintained the strain through 53 passes in the last two years. Occasionally amniotic passages have been made for experimental purposes. Any reference to this virus indicate a pool of infected allantoic fluid.

(2) 'James' - Strain: This strain has been isolated during the early stage of the present investigation in December 1950. The infected saliva was obtained from a young boy with bilateral parotitis within four days of the onset of the disease. After the original isolation and 3 further passages in the amniotic cavity, the strain has been adapted to grow inside the allantoic cavity. Since/

Since then it has been passaged twelve times through the allantoic cavity, during the last one and a half years. The virus contained in the allantoic fluid has been used for all experiments, unless otherwise stated.

(3) 'Paul' - Strain: The strain was isolated in this laboratory from another young boy with parotitis, in April 1951. Since the isolation in amniotic cavity, it has been possible to maintain it only by the amniotic passages. Attempts have been made to adapt it for growth in allantoic cavity; but so far it has not been possible. For experimental purposes only the amniotic fluid pools have been used.

All the three strains of the mumps virus has been maintained during the course of this investigation, by the passage of 0.1 ml. of a 10^{-1} to 10^{-3} dilution of the infected fluid in 8 day-old eggs and incubation at 35°C. The inoculated eggs are incubated for 5 days before harvesting the fluids.

Influenza:

(1) PR 8 - Strain has been maintained in this laboratory by allantoic passage in 10 day-old eggs. The inoculated eggs are incubated at 37°C and the infected fluids harvested on the 12th day.

(2) Lee - Strain is being maintained by allantoic passage/

passage in 12 day-old eggs. These are incubated at 35°C for 48 hours before harvesting the infected fluids.

New Castle Disease Virus (N.D.V.) of fowls:

'Herts' - Strain: This particular strain is highly fatal for the egg-embryos. It is passaged allantoically in 11 day-old eggs by the inoculation of 0.1 c.c. of 10^{-3} to 10^{-5} dilution of the infected egg fluid and the eggs are incubated at 37°C for 36 to 48 hours. Infected fluids are harvested within 48 hours of the inoculation.

Preparation of Virus pools and storage:

For the preparation of a pool of any virus strain, a large batch of eggs was inoculated by appropriate route in doses of 0.1 ml. The usual dilutions of the inoculations were 10^{-2} to 10^{-3} in case with the mumps and the influenza viruses and 10^{-4} with the N.D.V. After harvesting the fluids separately from each individual egg, they were tested for the presence of virus, in dilutions of 1:10 and 1:40 with fowl-erythrocytes, and for sterility. The/

The fluids were held at 4°C overnight and after the check for sterility the 'positive' fluids were pooled. After a light spin in centrifuge to remove any red cells and some cell debris or the urates, the supernatant was placed inside screwcapped glass vials and in sealed glass ampoules. The former have been stored at-36°C over solid CO_2 ice and the latter at -76°C in a mixture of solid CO_2 ice and absolute alcohol. During use, the virus in screwcapped vials was thawed, requisite amounts removed and the rest returned at-36°C again. The virus preserved at-76°C was never restored once the ampoules were broken.

Haemagglutination titration for the detection of Virus.

The terms 'titration for haemagglutination', and "titration of virus by haemagglutination test' have often been used synonymously in the text.

For the determination of the haemagglutinating capacity of any virus preparation, 0.25 ml. of a serial two-fold dilution was made inside small-bore "Wassermann" tubes (7 cms. x 1 cm.) using calcium saline as the diluent. Equal volume of a suspension of red cells, usually 0.5% was added to each tube, the mixture was thoroughly shaken and then allowed to settle at room temperature. The degree of sedimentation of the cells and their patterns of agglutination were noted according to the method recommended by Salk (1944). The end point of the haemagglutination titre (hereafter often abbreviated as H.A. titre) was taken as the final highest dilution of the specimen showing 50% agglutination (2+ reaction). Where a tube showed full dispersion of the cells (4+ reaction) in lower dilution and a complete absence of agglutination (0 reaction) in the next higher dilution tube, the 50% end point was the calculated intermediate value.

The results have been represented either as the reciprocal or the negative log value of the highest dilution taken as end point.

0.5%/

0.5% suspension of fowl erythrocytes has been used as a routine for the titration of the mumps and the influenza viruses. A similar suspension of group 'O' human erythrocytes was used for the titration of the Newcastle disease virus. When red cells from different species of animals were used for a comparative titration of the virus, they were all used in 0.5% suspension.

During the serial dilutions of virus preparations, separate glass capillary pipettes were always used. These pipettes were calibrated with the help of a "British Standard Wire Gauge'. When the cut ends of the pipettes corresponded with the No.11 hole of the gauge, they delivered 40 drops from a ml. of distilled water or saline (10 drops = 0.25 ml.)

Titration for Virus infectivity end-point:

Serial ten-fold dilutions of the virus infected egg fluids were made in 0.85% normal saline buffered at p^H 7.2 (0.025 M phosphate). For each dilution separate sterile calibrated capillary pipettes have been used. 0.1 ml. of the desired dilution of the fluid was inoculated into the allantoic or the amniotic cavity of eggs. For each dilution, batches of at least five eggs have been used for the inoculation. The age of the embryo, the time - period, and the temperature of incubation before/

before harvesting the fluids have been varied with the particular virus. The inoculated fluid from each egg was collected separately and tested for the presence of haemagglutinin. The end points for the 50% embryo infections dose (EID_{50}) were calculated by the method of Reed and Muench (1938). The values have been represented in log units per ml. of the neat infected fluid.

Titration of mumps haemolysin:

Following an earlier unpublished observation of Levens and Enders that haemolysis of chicken erythrocytes takes place when they are exposed to mumps virus at 37°C, their colleagues Morgan et al. (1948) made a systematic study on this haemolytic factor. Later Chu and Morgan (1950) modified their techniqueto titrate the haemolysin. A similar haemolytic property associated with N.D.V. has been described by Burnet and Lind (1950).

The methods of haemolysin titration as used by the above workers require large amounts of virus infected egg-fluids and were found unsuitable when the potency of the haemolysin was low. After a few preliminary trials the following method was found satisfactory and was adopted for the tests.

Two-fold serial dilutions of infected egg fluids were prepared in sterile isotonic phosphate buffer saline (p^H 7.0 to 7.2). To 0.5 ml. of each dilution was added an equal volume of 2% fowl cell suspension in the same diluent. The mixture was thoroughly shaken and placed in a refrigerator at 4°C. At the end of half an hour the degree of haemagglutination was noted by Salk's (1944) method and the tubes were gently shaken to re-suspend the red/

red cells. The test was further incubated for a total period of 3 hours. The tubes were shaken again and after a brief period at room temperature, they were placed at 37°C water bath. The incubation was continued for 16 to 20 hours with occasional shaking to re-suspend the cells. Each tube was centrifuged at 2,500 r.p.m. for 3 to 5 minutes to throw down the cells and the haemoglobin content of the supernatant fluid determined colorimetrically.

Along with each test two controls were also put up. In one 0.5 ml. volumes of serial dilutions of ammiotic or allantoic fluids from 14 day old normal eggs were mixed with equal volumes of cell suspensions; the other consisted of 2 tubes containing the buffered saline and an equal volume of the cell suspensions. A standard control to calculate the percentage of haemolysis was prepared by completely haemolyzing the cells contained in 0.5 ml. of the 2% suspension. For this, the cells were spun down and resuspended in exactly 1 ml. of distilled water containing a drop of N/10 ammonium hydroxide solution. Complete haemolysis takes place within a few minutes. The cellular stroma was separated by centrifugation and accurate two fold dilutions were made in distilled water.

The/

The haemoglobin content was estimated with a 'Spekker photoelectric absorptiometer' using violet filters (Ilford No. H601). The 'zero' point of the instrument was set against distilled water. About 0.5 ml. of the supernatant fluids from each tube were transfered to a micro-cell (No. H548 HILGER) and the direct readings on the scale were noted. The scale is so calibrated that the reading obtained is proportional to the logarithm of the amount of light transmitted through the solution; this bears an approximately linear relationship to the concentration of the colour.

The results of haemolysis titrations have been expressed in terms of the direct colorimeter readings. Where absolute values have been given to the particular haemoglobin concentration, the percentage was obtained by interpolating the values in terms of the percentage of haemolysis in control tubes.

Collection and Preservation of Sera:

Human Sera: Venous blood has been collected with aseptic technique. Whenever possible the sera were separated from the blood clots soon after collection. All the sera from infected patients were placed into small 5 ml. size glass vials or sealed in glass ampoules and stored in a cabinet containing solid CO_2 ice at-36°C. The human sera from other sources were either stored at-36°C or at 4°C, before they were examined.

Animal Sera: Blood samples were obtained by heart-puncture from the guinea-pigs, the mice and the rats under light ether anaesthesia. From the fowl, dog, cat and rabbit they were collected by veni-puncture. The sera were all stored at -36°C.

The sera from human and animal sources which have been stored frozen were treated after collection with a mixture of antibiotics in normal saline. The mixture contained 50,000 units of Sodium penicillin G and 50 mgm. Streptomycin sulphate per ml. One drop of the antibiotic solution (0.025 ml.) was added to each ml. of the serum.

When required for any particular test, all the frozen sera were thawed at 4°C, requisite amounts were pipetted out and the rest frozen again. The sera stored at 4°C had been heated at 56°C for 30 minutes and did not contain any preservative.

HAEMAGGLUTINATION - INHIBITION TEST (H.I. TEST).

Since Hirst (1942) and Salk (1944) outlined the methods for estimating the antibody content of human sera against influenza viruses, various modifications of their techniques have been described by other authors . These have varied depending on the source of the sera, the particular virus against which the antibody content was being titrated and on the type of the erythrocytes from different animal species as used for the test. An evaluation of this test for the detection of mumps anti-haemagglutinin in human sera was made by Robbins et al.(1949). Their method was re-examined in the present study besides those of others and was adopted with slight modifications.

Titration of the haemagglutinin:

The strength or the titre of haemagglutinin in a pool of virus infected extra-embryonic fluid was determined on the day of the serological test. The method was the same as that described earlier except that 0.85% sodium chloride solution was used as the diluent for the virus and for the preparation of the 0.5% fowl erythrocytes. The highest final dilution of the infected fluid showing 50% agglutination (2+) or the calculated dilution to show the same reaction was considered to contain

one haemagglutinating unit of virus per cubic centimeter. For use in the test, the infected embryonic fluid was diluted in normal saline so that each ml. contained 8 haemagglutinating doses.

Serological test:

Sera: All sera for the test were diluted 1:2 or 1:4 in normal saline and inactivated; the sera from experimental rabbits and mice were inactivated at 62°C for 20 minutes, and those from other animals and the human source were heated at 56°C for 30 minutes. If the tests had to be repeated on subsequent days, the sera preserved during the interval at 4°C were re-inactivated for 5 minutes at appropriate temperature. When several samples of sera were obtained from any source, they were all examined at the same time. If these were tested against more than one strain of the virus, serial dilutions were made in bulk and requisite amounts delivered in different series of tubes .

The tests were performed in "Wassermann" size tubes 7 c.m. x 1 c.m., with uniformly curved bottom. Two fold dilutions of the serum were made in normal saline in volumes of 0.25 ml. per tube. The initial serum dilution was usually 1:4., since inhibition at higher concentration was found to be due to normal serum factor. The range of dilution made/

made, was up to 1:128 when a low titre was expected, e.g. in normal human sera, in sera from patients collected in the acute stage of the disease and in the pre-inoculation or control samples from animals. The dilutions were extended to 1:1024 where higher titres were expected, e.g. in convalescent human sera or the sera from animals subsequent to inoculations with the virus. 0.5 ml. of the diluted virus, containing 4 units, were added to each serum dilution tube. The tubes were shaken for thorough mixing. The mixture was incubated at 37°C in a waterbath for one hour. At the end of the period 0.25 ml. of 0.5% fowl erythrocyte suspension (the same as used to titrate the haemagglutinin) was added to each tube. After thorough mixing, the test was allowed to stand at room temperature for about 30 to 45 minutes. The reading was taken in the same manner as for the haemagglutinin titration.

The end points are usually sharp. The anti-haemagglutinin titre of the serum is considered to be the dilution of the serum which completely inhibited or produced at least 50% inhibition (2+ reaction) of agglutination by the virus. All titres have been expressed either as the reciprocal or as the negative log value of the serum after all the reagents were added. In cases where the limit between the agglutination and the inhibition was not sharp and/

and more than one tube showed 50% inhibition, the tube with higher dilution was taken as representing the end point.

In each test, the following controls were always included.

(1) To see the action of the serum alone on the fowl cells: into a tube containing 0.25 ml. of the lowest dilution of the serum, aliquots of normal saline and cell suspension were added; this tube should not show any dispersion of the cells.

(2) To see the haemagglutinating activity of the virus: into three tubes, 0.5 ml., 0.25 ml. and 0.125 ml. of the virus dilution was placed; enough saline was added to make the volume up to 0.75 ml. After adding the cells, the mixture was allowed to settle at room temperature. The tubes contained 2, 1 and $\frac{1}{2}$ unit of the virus in that order. In a satisfactory test, the first two tubes showed full (4+) agglutination and the last one at least a partial (2+) reaction.

(3) To see the stability of the fowl cells in the saline: 0.25 ml. of the fowl cell suspension was added to 0.75 ml. of normal saline; this tube should always show a complete negative reaction.

(4)/

(4) To see the specificity of the test and compare it with the results of earlier tests: a normal human or pre-inoculation sample of animal serum and a convalescent human or immune anti-serum of known titre were also put up like the test proper upto suitable dilutions. In no case should the titre of these control sera vary by more than two-fold dilution from that of the earlier results.

During the titration of human sera against the Newcastle disease virus, the method was slightly modified. Group 'O' human cells were used to titrate the virus. The virus-cell mixture (titration of the virus) and the serum - virus - cell mixture (the test proper) were allowed to stand at $4^{o}C$ before reading the results. The readings were taken before all the cells settled at the bottom of the tube, because otherwise the virus elutes even at this low temperature and false negative results are obtained.

H.I. test for the identification of Virus: For this purpose the technique of the test has been the same, except that a fixed dilution of the virus was added to serial dilutions of known convalscent serum and normal sera.

COMPLEMENT - FIXATION TEST.

The complement fixation test (C.F. test) has been employed in the present study for several purposes. They were as follows:

(i) to determine the presence of antibody against the mumps virus in human sera from apparently 'normal' persons and from infected patients;

(ii) to assess the immunity which may result from the inoculation of experimental animals with the virus;

(iii) to detect the multiplication and growth of the virus in the tissues of inoculated animals or in the fluids and the membrances of inoculated embryonated eggs, and

(iv) to distinguish any difference that may be present amongst different strains of the mumps virus.

In a limited series of experiments the test has also been utilised to define the serological relationship of the viruses of mumps and Newcastle disease.

A multitude of procedureshave been employed by different workers for this test. It is thus rare to find any two of them identical so that the results are never 'strictly' comparable. In the light of the recent knowledge of the two different complement fixing antigenic components which could be/

be identified in the infected embryonated eggs, the methods advocated by other workers were re-examined; the observations have been detailed in chapter III . The technique outlined by Enders and Levens (1948) has been followed in the general lay-out of the test and the procedure detailed below was finally adopted.

Sera:

The sera were diluted 1:2 or 1:4 in normal saline before inactivation. The human and the guinea-pig sera were heated at 56°C for 30 minutes and those from other animals were heated at 62°C for 20 minutes . They were stored in the refrigerator at 4°C for 2 to 24 hours and re-inactivated at appropriate temperature for 5 to 10 minutes. This second heating is said to remove the anti-complementary property of some sera and was found to be extremely useful,especially with the rabbit sera.

When more than one sample of serum was obtained, they were all titrated simultaneously with the same antigen and the same complement dilutions.

Complement:

The sera from at least four guinea-pigs were collected, pooled and stored at-36°C contained in sealed glass ampoules. There is usually no diminution in the titre for at least 2 months (Enders and Levens, 1948). However, on no occasion the/

the guinea pig sera have been used as complement after 3 to 4 weeks. The complement was titrated on the day before the test by the following method.

About 2 ml. of a 1:60 dilution of the guinea-pig serum was prepared in normal saline (0.85%). Into a series of Wassermann tubes volumes of 0.20 ml., 0.19 ml., 0.18 ml., 0.17 ml., 0.16 ml., 0.15 ml., 0.14 ml., 0.13 ml., 0.12 ml., 0.11 ml., and 0.10 ml. of the above dilution were pipetted, so that they were placed only on the rounded bottom of these tubes. The volumes were each brought up to 0.5 ml. by the addition of normal saline, and 0.25 ml. of sensitized sheep cells was added. The contents of the tubes were thoroughly shaken and incubated in the waterbath at 37°C for 30 minutes. The amount of complement in the last tube with complete haemolysis was taken as one unit. If any pool of guinea pig serum did not show haemolysis even in a dose of 0.20 ml. of 1:60 dilution, it was discarded. For use in the test, the complement was diluted so that 0.3 ml. contained 2 units.

Haemolytic System:

Sheep cells preserved with 0.1% formal-dehyde (Burroughs Wellcome & Co.) were used. They were washed 4 to 5 times in normal saline and finally/

finally packed in an angle head centrifuge at 2,500 r.p.m. for 20 minutes. The supernatant was pipetted off and to half the volume of the requisite amount of cell suspensions required for the test, enough packed cells were added to make a 2% suspension.

Glycerol preserved (Burroughs, Wellcome & Co.) anti-sheep rabbit serum, with a titre of 1:800 to 1:1000 was used as the amboceptor. To the 2% sheep cell suspension an equal volume of a dilution of the rabbit serum containing 2 units of amboceptor was added 15 minutes before the titration of complement and the mixture incubated in a 37°C water bath. The haemolytic system thus contained 1% sheep cells and 1 unit of amboceptor and was used in a volume of 0·25 ml.

After titration of complement, the sensitized cells were stored overnight at 4°C; before being used for the test-proper on the next day, they were allowed to come to room temperature and then warmed again in the water bath at 37°C for 15 minutes.

Antigens:

For the titration of antibody against the mumps virus, two different kinds of antigens - 'Viral' (V) and 'Soluble' (S) antigens, have been used. The viral antigens were prepared from the allantoic fluids infected with the 'Enders' or 'James' strains and the amniotic fluid infected with the 'Paul'/

'Paul' strain. The soluble antigens were prepared from the suspensions of the C.A. membranes infected with 'Enders' and 'James' strains only. The methods of the preparation of these antigens and their standardisation have been described in Chapter III . For use in the test appropriate dilutions of these antigens in normal saline were used in doses of 0.1 ml.

Procedure employed for the complement - fixation test to detect the antibody.

Two-fold serial dilutions in saline of the inactivated sera were prepared, beginning with 1:2 or 1:4 dilution in a volume of 0.25 ml.; for sera where negative or low titres were expected the dilutions were made up to 1:256 or 1:512 and in convalescent specimens they were made up to 1:2048. Into two series of tubes 0.1 ml. of each dilution was pipetted; 2 units of the diluted complement in a volume of 0.3 ml. was added to each tube and then 0.1 ml. of appropriately diluted antigens - the viral and the soluble antigens, were added in separate series. These antigens had been previously standarised. The mixtures were shaken and incubated at 4°C overnight. On the following morning (after about 12 to 16 hours) after the mixtures were allowed to come to the room temperature, 0.25 ml. of sensitized sheep's cells were added to each tube. The test was incubated for ½ hour at 37°C in a waterbath

after which the readings were recorded. The last dilution of the serum giving complete or 50% fixation of the complement was considered to be the end-point. All antibody titres have been recorded as the initial dilution of the serum.

Besides the test proper, for each serum the following controls were included. Three sets of dilutions from 1:2 to 1:16 were prepared in volumes of 0.1 ml; into one series was added normal saline and into the others the normal viral and soluble antigens, in volumes of 0.1 ml. These were completed as the test proper. The tubes containing normal saline served to check the anticomplementary behaviour of the serum and the others detected any non-specific reaction between the serum and the antigens.

To test the sensitivity of the particular batch of tests and the reproducibility, known positive convalescent mumps serum and negative sera were always tested each day along with the test proper.

In spite of previous titration of the complement, its potency after overnight incubation at 4°C was tested. Into two tubes containing 2 units and 1 unit of the diluted complement saline was added to bring the volumes up to 0.5 ml. in each. After the addition of the sensitized cells, the progress/

progress of haemolysis was noted during the half-an-hour period of incubation at 37°C. In any satisfactory batch of tests, the tubes with 2 units of complement showed complete haemolysis within 5 to 10 minutes; the tube with 1 unit of complement showed more than 50% haemolysis at the end of 20 minutes and complete haemolysis by the end of 30 minutes when the results were recorded.

Other controls included in each test, were those to see the effect of saline on cells and the anticomplementary behaviour of the two antigens - viral and soluble at their 'working dilution'.

Criteria for recording the test:

Any batch of test was considered unsatisfactory if any of the following points were noted:

(1) The complement control tube containing 2 units didnot show complete haemolysis within 30 minutes or the tube containing 1 unit showed complete haemolysis with 5 minutes (the former result indicated deterioration of the complement or absence of sufficient amount for the test, and the latter indicated its presence in excess).

(2) The antigen control tubes did not show complete haemolysis within 10 minutes.

(3) The "normal" viral and soluble antigens fixed any of the test serum above 1:4 dilution.

The/

(4) The titre of the convalescent serum (positive control) showed more than 2-fold difference in titre than that previously estimated.

(5) The titre of the 'normal' serum (negative control) showed fixation above 1:4 dilution

Any serum which showed anti-complementary property was tested again after re-inactivation. Usually this property was thereby considerably diminished or disappeared altogether. On some occasions, however, the same range of the anti-complementary titre was noted in the repeat test. In these cases the result of the test has been recorded only when the c.f. titre of the serum was found to be higher than that of the anti-complementary titre.

Complement fixation test with other antigens:

Some of the human sera, were set up with Newcastle disease virus antigen. For this purpose a pool of allantoic fluid infected with the virus has been used. No attempt was made to standardise it by "Box-titration". The crude fluid had a high H.A. titre and it was used in 1:4 dilution. For all the tests the same antigen which had been stored at $4^{o}C$ was used. The method was the same as was used with the viral antigen of mumps.

Complement fixation test for measuring the antigenic content of egg fluids:

In principle the antigen content of any virus infected fluid or tissue emulsion was titrated against mumps antibody of known titre.

Serial two fold dilutions were made in saline using 0.1 ml. as unit volume. The dilutions started from 1:2. Equal volumes of a suitable dilution of known high-titre (convalescent) serum was added and the test completed as before. Readings were recorded similarly. The highest dilution of the antigen showing a complete or at least 50% fixation was taken as the end point. Controls for any non-specific fixation has always been made by setting three lower dilutions of the antigen against known negative serum.

Notation used to record the degree of fixation:

4+ or 4	=	Complete haemolysis or no fixation.
3+ or 3	=	More than 50% haemolysis of cells or less than 50% fixation.
2+ or 2	=	50% haemolysis or 50% fixation.
1+ or 1	=	Slight haemolysis or more than 50% fixation.
0	=	No haemolysis or complete fixation.

(Trace) Tr, (> or <), denote grades of fixation intermediate between those mentioned above.

Titration of Neutralizing Capacity of Serum:

Two-fold serial dilution of serum was made in sterile normal saline (buffered at P^{H} 7.2 with 0.05 M. phosphate) containing penicillin and streptomycin sulphate at concentrations of 500 units of each per ml. The dilutions were made using separate calibrated glass capillary pipettes. Each dilution of the serum was mixed with an equal volume of fixed dilution of infected allantoic or amniotic fluid. The dilution of the infected fluid was also made in the same diluent as above and the dose of the virus varied according to the particular experiment. The serum-virus mixture was incubated at room temperature (24 to 26°C) for 1 hour before inoculation into the 8 day-old eggs through appropriate route. Each egg received 0.1 ml. of the mixture and 3 to 6 eggs were inoculated for each dilution. After incubation at 35°C for 5 days the inoculated allantoic or amniotic fluids were tested individually for the presence of haemagglutination at dilutions (final) of 1:10 and 1:40. Positive or negative results of haemagglutination indicated the infection or protection of the embryo. Embryo protective dose (EPD_{50}) was calculated as the dilution of serum that would protect 50% of embryos against the virus as determined by the method of Reed and Muench (1938).

/All

All titres of serum are expressed as reciprocals of final dilutions after adding the virus.

With some paired sera where both the acute and convalescent samples were titrated at the same time against the same fixed dilution of the virus, the number of eggs inoculated for each serum dilution were sometimes less. In these cases the titre of the serum has been expressed as the reciprocal of the final dilution at which 50% or more embryos are protected. This has been differentiated from E P D_{50} by recording as the neutralization titre (N T_{50}).

CHAPTER III

THE STANDARDISATION OF METHODS.

During the course of the present study several methods were employed to titrate the virus content of infected fluids and to determine the levels of the specific antibodies in sera of man and experimental animals. Various workers, in the past, have described different techniques for these tests. Some of these were re-examined and found suitable after modification.

The methods finally adopted have been mostly outlined in the previous chapter. For the sake of convenience two other techniques have been included in this chapter. Preparation of the test antigens, their standardisation and the significance of the results of different tests will be discussed. Relevant experimental observations which were responsible for the selection of different methods will also be described briefly.

Haemagglutination Test.

a). Diluent:

Burnet and other Australian workers recommend the routine use of normal saline containing 0·1% $CaCl_2$ and buffered (boric acid salts) at pH 7·2 for the titrations of virus. Although their method has been employed during the present investigations, no significant variation in titre could be observed when ordinary saline (0·85% Na Cl) or saline buffered with phosphate salts at pH 7·2 to 7·4 were used for comparative titrations.

b). Temperature:

The end point of the titre of virus was found to be modified by the temperature at which the red cells were allowed to settle. Usually there was no difference in titre when the virus-red cell mixture was left at 4°C and at room temperature. It was however found that doubtful or false positive haemagglutinations at room temperature were eliminated when the test was re-shaken and allowed to re-settle at 4°C. When the mixture was incubated at 37°C the titre was always higher by 2 or 4 fold dilutions. This will be further evident from the experimental results in Chapter IV (A).

c)./

c). Erythrocytes:

The use of red cells from different species of animals or from human beings resulted in a significant difference in the titres (Chapter V (A)). But even with the same type of red cells the titre of the H.A. test varied inversely with the strength of the cell suspension. The results of one of the tests with different strengths of fowl cell suspensions are shown in the following table:

Cell-suspension.	Dilution of the virus (reciprocal)					
	40	80	160	320	640	1280
5%	4	3	0	0	0	0
2%	4	4	4	0	0	0
1%	4	4	4	4	0	0
0.5%	4	4	4	4	2	0
0·025%	4	4	4	4	4	4

4 = Complete agglutination.
0 = No agglutination.

This result shows that it is possible to obtain higher titres when a lighter suspension of cells is used for the test.

To obtain uniform cell suspensions on different days of the test, reliance was placed mostly/

mostly on the speed and time of centrifugation needed to pack the red cells. Further, the packed cells were carefully measured with micro-pipettes and a limited set of glassware was used for the purpose. Occasionally, the densities of different suspensions were checked by counting the red cells with a haemocytometer.

d) Dilution technique:

Since the serial dilutions of the virus were made in small volumes (0·25ml.) and inside small bore test tubes, it was felt that the use of ordinary graduated pipettes might result in a greater loss of the virus. For this reason, in all the titrations, glass capillary pipettes calibrated to deliver a fixed volume (40 drops to a ml.) were used. Comparative titrations of a pool of virus showed wider variation of the end points when ordinary graduated pipettes were used. The efficacy of the capillary pipettes for preparing the dilutions will be evident below.

It was found that the haemagglutinin of mumps virus is rapidly destroyed at temperatures higher than 60°C. Two or three rinsings of the used capillary pipette in boiling water or saline removed all the traces of the haemagglutinin.

It/

It was therefore possible to use a limited number of capillary pipettes, carefully calibrated, and quickly free them of the haemagglutinin by the above method. In fact, it was found that the same pipette could be used to prepare serial dilutions provided it was cooled after rinsing in boiling saline (by drawing in and out cold normal saline and blowing out the last drop carefully).

e). Pipetting error:

Determination of the pipetting error gives an idea of the significant variation in titre that may occur in a particular batch of the test. A pool of infected allantoic fluid was titrated 20 times on the same day using the same fowl cell suspension. The results are shown in table I appendix (A). In 25% cases there was a two-fold variation in the titre. The variation was less marked when the 50% end point (2+ reaction) was calculated by interpolation.

f) Comparison of the results of the test performed on different days:

Two different pools of virus were titrated for five consecutive days, using the same batch of/

of fowl cells preserved during intervals at 4°C. The cell suspension was prepared fresh on each day. The end point varied within two fold dilutions in all cases except one where a four fold difference was observed.

TITRATIONS FOR THE INFECTIVITY OF THE VIRUS:

Various diluents such as serum-saline mixtures, nutrient broth etc., have been recommended by different authors for use during the infectivity titrations. No attempt was made to investigate the relative usefulness of different methods. In the present study, all titrations for infectivity of the virus or for the protective capacity of serum against the virus, were carried out using sterile phosphate buffered saline as the diluent.

Reproducibility:

A pool of mumps virus stored at -76°C was tested for EID50 dose, on four occasions within 1½ months. The negative log values in these cases were 4·6, 4·85, 4·25 and 4·05. The variation in the first three titrations were within the limits of the experimental error, specially when 5 to 6 eggs were used for each dilution (Knight, 1944a). The slightly lower value in the fourth case is possibly due to storage conditions.

Haemagglutination - Inhibition test:

(a) Antigen:

The pooled extra-embryonic fluids containing the appropriate strains of the virus were stored at -36°C. On each day of the test the haemagglutinins were tested for the potency of the antigen with a particular batch of fowl cells. Preliminary to the titration, the fluid, especially the allantoic fluid, was incubated at 37°C water bath for about ½ hour. As a result, any virus which remained adsorbed on the urate deposits were freed and became available for a more satisfactory titration of the haemagglutinin.

The end point was always determined by calculating the 50% or 2+ reaction. This step was found particularly important because of the close co-relation between the titre of the H.I. antibody and the amount of the virus used as antigen (table II, appendix (A)). Two fold increase in the amount of the added virus caused a corresponding reduction in the titre of the antibody content. It was also observed that with high titre fluids a lower titre (two fold) of the antibody is obtained when the virus dilutions are calculated on the basis of complete or 4+ haemagglutination.

(b) Serum: It has been mentioned already that the sera were inactivated at different temperatures depending on the source. Re-inactivation of the sera/

sera were carried out during repeat titrations and during the intervals they were stored at 4°C or at -36 C. The re-inactivated sera (not exceeding 5 times) stored at 4°C did not show any significant decrease (more than 2 fold dilution) in the titre over a period of 3 weeks.

(c) Technique of the test:

The results of the test were more satisfactory when the sera were diluted with a graduated capillary pipette instead of the ordinary pipette. With any particular batch of sera all the dilutions were made with the same pipette. It was thoroughly washed in a bath of boiling saline after preparing each serum dilution.

Similar to the earlier observations made by Robbins et al. (1949), the titre of a serum did not show a significant difference when the virus-serum mixture was incubated for 1 hour at 37°C or for overnight at 4°C. It was however found that if the red cell suspension was added immediately after the virus was mixed with serum, the test tended to show a higher titre (table II, appendix A).

(d) Significance of the difference in titres of the test performed on the same day:

Doubling dilutions of a serum were prepared in fifteen separate series. The tests were then completed as usual, using the same batches of the diluted virus and the cell suspension (table III appendix A).

The/

The titrations did not show more than a two-fold difference in titre. Even a two fold difference was observed in only 2 cases. There may be, however, a difference in the degree of reaction at a particular titre, that is, the same sera may show a complete (0), partial (1+) or 50% (2+) inhibition of haemagglutination. For this reason the difference in the degree of reaction in sera showing the same end point, has not been considered significant.

During the titration of serial samples of sera from experimental animals or from mumps patients it was observed that the titre of the successive sera differed by a two-fold dilution in many instances. A uniform rise or fall in titre was apparent. Consequent to the greater accuracy of the antibody titration by the use of capillary pipettes it is probable that the two fold difference in titre in sera referred above protrays an actual but small difference in the antibody content.

(e) Significance of the difference in titres of sera tested on different days:

When a convalescent serum was tested on different days with the same batches of virus and fowl cells, a two fold difference in titre could be observed (3 out of 7 cases). The two fold difference in titre was more pronounced when different batches of virus and of fowl cells were used (5 out of 7 cases); moreover, a four fold difference/

difference was noted in one case.

In order to achieve a better reproducibility of the result, the following precautions were undertaken:

(i) Known convalescent sera were always included in any batch of test. If there was more than four fold difference in titre from that of the previous day, the test was repeated.

(ii) All the sera from a particular experiment were tested in one or two batches using the same red cells and the virus.

COMPLEMENT - FIXATION TEST

I. PREPARATION OF THE ANTIGENS FOR COMPLEMENT FIXING TESTS.

A. VIRAL ANTIGEN.

A large batch of 8-day old eggs were inoculated with 10^{-1} to 10^{-3} dilutions of pooled allantoic fluid infected with the Enders or the James Strains of the mumps virus. Each egg was inoculated with 0·1 ml of the appropriate dilution into the allantoic cavity. The eggs were inoculated at 35°C for 5 to $5\frac{1}{2}$ days. The allantoic fluid from each egg was tested for the presence of the haemagglutinin at 1:10 and 1:40 dilutions. The positive (infected) allantoic fluids were pooled and clarified at low speed (2,500 r.p.m. in an angle head centrifuge for 10 - 15 mins.) to remove the cellular debris and urate deposits.

A concentrated mixture of antibiotic solutions (streptomycin and penicillin), each at 50,000 units per ml.) was added to the pooled clarified allantoic fluid, so that each antibiotic was present in a strength of 500 units per ml. The fluid was placed inside a sterile cellophane bag and dialysed against 30 to 40 volumes of sterile phosphate buffer solution (p^H7·2). The dialysis was carried out at 4°C. The buffered saline was changed once during the time.

The efficacy of the dialysation was usually judged as follows. The dialysed fluid is frozen (at - 36°C)/

(at -36°C) and thawed for two or three times. If the dialysation is unsatisfactory, small granular deposits of the urates appear in the fluid and do not go into solution even on repeated shaking. When, however, the antigen is satisfactorily dialysed, the fluid presents a uniform turbidity with greyish-blue opalescence.

For preparation of the viral antigen from the Paul Strain of the mumps virus, the eggs were inoculated with pooled amniotic fluid through the amniotic cavity. The positive fluids were pooled and used as antigen, after a clarifying spin. The amniotic fluid pool was not dialysed.

The infected fluids, that is, the antigens were placed inside sterile screw capped glass vials or glass ampoules and stored at -36°C until used.

The majority of the viral antigens used during the present study were uninactivated. In some cases the inactivation was carried out by irradiating the infected fluid with ultraviolet light. The method of irradiating the antigen was same as that described in chapter VI .

B. SOLUBLE ANTIGEN.

The soluble antigen was prepared only from the eggs inoculated with the Enders and the James Strains. The C.A. membranes from the infected eggs (see above) were pooled. They were washed repeatedly in several

changes/

changes of sterile normal saline to remove the adherent streaks of urates, yolk, albumin or the blood clots. The membranes were placed in between the folds of sterile filter papers to remove the fluid as far as possible. They were placed in sterile glass vials and the weight in grams was measured accurately.

The membranes were then placed on petri dish and cut into small pieces with sharp pointed scissors. They were thoroughly emulsified in TenBroeck's tube and enough normal saline was added to make a 20% suspension. The suspension contained inside a screw-capped glass vial, was subjected to alternate freezing (at -76°C) and thawing (37°C) for at least 4 or 5 cycles to rupture the cells of the C.A. membrane. It was clarified at 2,500 r.p.m. (angle head centrifuge) for 30 mins. The supernatant fluid (low speed supernate of the soluble antigen) was then subjected a high speed centrifugation in a bucket type 'Ecco' centrifuge. It was spun (at room temperature) at 15,000 r.p.m. for 1 to 1¼ hours, when the virus haemagglutinins were thrown down. The supernatant fluid, usually with a reddish tinge, was used as the 'soluble' antigen. It was preserved and stored in the same way as the viral antigen.

In/

In some cases, the soluble antigen was prepared by using 0·08% sodium azide solution in normal saline. The latter was used to wash the membranes as well as to make the 20% suspension. No antibiotic solution was used as preservative for these batches of the antigen.

Both the viral and the soluble antigens were standardised against known anti-serum to determine the optimum dilution at which they were to be used for diagnostic tests. Sometimes when the soluble antigen is held at 4°C for several weeks, there may be some precipitate. These can be removed by a low speed centrifugation. The titre of the soluble antigen is not appreciably altered thereby.

2. TITRATION OF ANTIGENS

During the preliminary stages of this study the antigens were titrated against paired samples of sera from known mumps patients. Later, anti-sera from rabbits were used for the titrations. The potency of the antigens were determined by two methods:

(I) 'Box-titration'.

(II) Comparison of the C.F. property of different batches of antigens with that of the test antigen/

antigen.

I) Doubling dilutions of the antigen were prepared and distributed in a series of test tubes in 2 batches. Serial dilutions of the acute (or normal) and convalescent sera were also prepared and these were added to diluted antigens. The technique of the test has been described in the earlier chapter. From the diluted antigens another batch of test was arranged to denote any anti-complement-:ary behaviour of the antigens.

The following table shows an actual titration of a batch of viral antigen. Titrations of the paired sera with normal antigens (prepared similarly from uninoculated eggs) were also included as controls.

Dilution of antigen	Dilution of convalescent serum (reciprocal).							Dilution of Acute serum (reciprocal)				Compl. contr.
	4	8	16	32	64	128	256	4	8	16	32	
1/2	0	0	0	0	0	0	4	0	4	4	4	3
1/4	0	0	0	0	0	2	4	2	4	4	4	4
1/8	0	0	0	0	0	2	4	4	4	4	4	4
1/16	0	0	0	0	3	4	4	4	4	-	-	4
1/32	0	0	0	4	4	4	4	4	4	-	-	4
1/64	0	2	4	4	4	4	4	4	-	-	-	4

(0 - complete fixation: 4 - no fixation)

In some cases normal antigens, especially the viral antigen, may show non-specific fixations with either or both the acute and convalescent sera. This/

This, when present, is manifested in the lower dilution tubes (neat, 1:2 or 1:4). The test antigens were occasionally anticomplementary. For these reasons, the antigens have never been used undiluted. The particular antigen (viral) which showed the above result of titration, was used at a dilution of 1:4.

II) In the second method of titration, the test antigen together with the newly prepared antigens were titrated against fixed dilutions of the con-:valescent (1:8) and of the acute or normal (1:4) sera. The antigen or the antigens which showed comparable titre with that of the test antigen were selected, provided it did not show fixation of the complement in presence of normal serum (1:4) and was devoid of anticomplementary property. The following table gives an example. -

Antigen	VS.convales.serum (1:8) Antigen dilution ⊠ 4	8	16	32	64	128	256	512	VS.acute serum(1:4) Antigen dilution 4	8	16	32	Comple-:ment control 2	4	8
I	×0	0	0	0	0	0	0	3	0	2	4	4	0	4	4
II	0	0	0	0	0	4	4	4	4	4	4	4	4	4	4
III	0	0	0	4	4	4	4	4	4	4	4	4	4	4	4
Test Antigen	0	0	0	0	2	4	4	4	2	4	4	4	4	4	4

⊠ - reciprocal of initial dilution.

×0 - complete fixation of complement.

4 - no " " "

Of the three antigens, No. II appears more satisfactory/

satisfactory than the test antigen because it does not show any fixation in presence of acute serum at 1:4 dilution. It may be used at the same dilution as the test antigen. Antigen No. I shows non-specific fixations and is also anticomplementary at lower dilutions, but has a higher titre of the complement fixing property. This type of antigen can also be used after suitable dilution at which the titre becomes comparable to that of the test antigen and the non-specific fixation or the anti-complementary property is not manifested.

After the selection of the antigen by this method, further check may be made by box-titrations. This method was also used to determine the potency of the antigens from time to time after storage at 4°C or at -36°C. The dilution of the antigen used in tests was one half of that determined as the end-point in the box-titration.

3. DIALYSATION OF VIRAL ANTIGENS.

Dialysis did not result in any difference in the titre of the complement fixing antigen. The antigens when tested for the content of haemagglutinins sometimes showed a two-fold reduction in titre.

Occasionally, undialysed viral antigens showed peculiar reactions during the titrations against known convalescent serum. It was specially marked in some antigens prepared from freshly harvested allantoic fluid or when the latter was stored

at/

at -76°C for long time. There was incomplete fixation in several of the lower dilution tubes followed by complete fixation at higher dilutions (see table below).

Antigen	Antigen dilution (reciprocal)														
	Vs. Convalescent serum								Vs. normal serum			Compl. contr.		
	2	4	8	16	32	64	128	256	2	4	8	2	4	8
No. I	0	0	0	0	0	4	4	4	4	4	4	4	4	4
No. II	1	2	Tr	0	0	0	1	4	4	4	4	4	4	4
No. II (dialysed)	0	0	0	0	0	0	2	4	4	4	4	4	4	4
Test antigen (dialysed)	0	0	0	0	0	0	0	4	2	4	4	2	4	4

0 = Complete fixation of compl.

Tr, 1, 2 = Incomplete " " "

4 = No fixation " "

Not infrequently, the antigens showing this peculiarity also contained haemagglutinins to a high titre. The effect was entirely eliminated by heating the antigen at 56°C for 30 mins. Dialysation of the fluid followed by storage at 4°C for 2 or 3 weeks was also found helpful in eliminating this reaction.

It is difficult to find an explanation of this phenomenon without further investigation; it seemed possible that the haemolysin of mumps virus was responsible, but the absence of the reaction when tested/

tested against normal serum was somewhat puzzling.

4. Soluble antigen:

During the preparation of the soluble antigens it was noticed that the washings of the C.A. membranes did not reveal the presence of haemagglutinins (negative at 1:2). Some amounts of the same were however liberated in the emulsified suspension. The released haemagglutinin, obviously, was derived either from the adsorbed virus or from the cell contents.

Most of the haemagglutinin could be removed by centrifugation at 15,000 r.p.m. for 1 to 1¼ hours (see also chapter IV) leaving the titre of the complement fixing antigen unchanged. This will be evident from the two following tables. Moreover, it was found that as a result of the high speed centrifugation non-specific fixation of the complement by C.A. membrane suspensions could be greatly diminished. The responsible factor was detectable in the deposit of the high-speed centrifugation.

Similar non-specific fixations were also noticed with crude normal C.A. membrane emulsions.

H.A. Test:

Fraction	Dilution of the fluid (reciprocal)							
	2·5	5	10	20	40	80	160	320
Crude C.A. membrane suspension	4	4	4	4	2	0	0	0
Soluble antigen	4	2	0	0	0	0	0	0
Deposit*	-	-	4	4	4	4	4	2

* (resuspended in 1/10th original volume)

C.F. Test:

Fraction	Dilution of antigen (reciprocal)													
	Vs. cont. serum						Vs. acute serum				Compt. cont.			
	5	10	20	40	80	160	5	10	20	40	2·5	5	10	
Crude C.A. Mem. Susp.	0	0	0	0	4	4	1	4	4	4	2	4	4	
Soluble antigen	0	0	0	1	4	4	4	4	4	4	4	4	4	
Deposit	0	0	0	0	0	0	0	0	0	3	0	1	2	

0 = complete fixation 4 = No fixation

5. Storage of Antigens:

The titre of the viral or the soluble antigens has been found to be well maintained at -36°C for at least 6 months. Two batches of antigens when tested against the same antiserum after 13 months did not show more than a two fold reduction in their titres.

At 4°C no reduction in titre has been observed when antigens were tested after 2 to 3 months.

Usually, the antigens were stored at -36°C and small quantities were removed and stored at 4°C in between the tests.

6. Antibody titrations by C.F. test:

(a) Reproducibility of the test (same day):

Because of the small amount of reagents used for the test, the dilution of the serum or the addition of antigens were carried out with the help of graduated capillary pipettes. Usually, the same pipette was used for the serum dilutions.

With/

With a particular batch of the diluted antigen (viral), the complement and the sensitised sheep cells, a pool of convalescent serum was titrated 15 times. It was found that only in 2 instances there was a two-fold difference in the titre. In others the titres were the same, although slight differences in the degree of reaction could be observed. The results are shown in tableIV, appendix A.

(b) Re-producibility of the test on different days:

A viral and a soluble antigen were each tested against the same convalescent serum on 6 different days. Two different batches of sheep cells were used for the tests.

With the soluble antigen a two-fold difference in titre was observed only on one occasion. With the viral antigen a two-fold difference in titre occurred on one day and a four fold difference on another day.

The precautions taken during the performance of the test on different days were the same as that described in connection with H.I. tests.

(c) Incubation of the test:

When the serum - antigen - complement mixture was incubated for 1 hour at 37°C and not for overnight at 4°C, higher titres were always evident. But in the former method non-specific fixations in lower dilution tubes were more frequent and the results/

results were not clear cut as in the latter method.

(d) Sensitised cells:

The cells were prepared in bulk and after the titration of the complement were stored overnight at 4°C for use in the test next morning. Before use they were brought to room temperature and incubated for 10 minutes.

Comparative titrations with sensitised cells where the diluted haemolysin and the cell suspension were stored separately and mixed together before the completion of the test next day, did not show any difference in the results.

NEUTRALISATION TESTS:

With any batch of sera obtained from mumps patients or from animals after experimental inoculations, the source of the test-virus was usually the same pool of infected allantoic or amniotic fluids. For this reason the results obtained with individual or pooled sera in connection with any experiment are more comparable.

The test virus was stored at -76°C till all the sera from the particular experiment were tested. The decline in infectivity titre during the period of storage has been mentioned in appropriate chapters.

The titrations for the reproducibility of the test were carried out on two occasions. The results (EPD50) have been shown in table V appendix A.

For technical reasons it was not possible to perform the neutralisation tests with human sera using large numbers of eggs (5 to 6) at each dilution. An alternative method was formulated whereby the serial samples of sera from an individual would show differences in titre even when smaller number of eggs were used for each dilution of the serum.

After a few preliminary trials it became evident that a marked difference between the titres of acute and convalescent samples could be demonstrated when they were titrated against a minimum amount of the virus/

virus. 50 to 100 EID50 doses of virus were found sufficient to infect all the inoculated eggs and have been used to test the human sera.

The results of titrations of two convalescent sera where in one case 5 to 6 eggs were used for inoculations at each dilution and in the other where smaller number of eggs were used, are shown in table VII appendix A. From the latter it will be observed that if the serum dilution which protected all or at least 50% of the inoculated eggs (NT50) is taken as the end point, there is some correlation with the EPD50 dose.

It should be pointed out that due to simultaneous titrations of the paired sera or serial samples of the sera it was possible to demonstrate the increase (fold-rise) or decline of the levels of the neutralising antibody. Since the same virus pool was used to titrate all the sera, the results from individual patients are comparable. Moreover, as will be shown in a subsequent chapter, the time of appearance of the neutralising antibody or its rise and fall after the onset of infection could be correlated with those of other antibodies. It seems that although the method does not conform to the accepted standards of the neutralisation tests a fair indication of the variation in the antibody level could be demonstrated.

The/

The period and the temperature of incubation of the serum-virus mixture were according to the method described by Leymaster and Ward (1949). No attempt was made to investigate the usefulness of the alternative methods usually employed for other viruses.

During the present study it was observed that infected allantoic fluids when tested undiluted against fowl cell suspension may show absence of haemagglutination, although a positive reaction was evident on further dilution. For this reason each allantoic fluid was tested at two dilutions e.g. 1:10 and 1:40. A fluid was considered positive when at least one of the dilutions showed positive haemagglutination.

THE TREATED CELL AGGLUTINATION TEST (BURNET'S TEST).

A. Preparation of the virus-treated red cells.

Considerable difficulties were experienced during the preparation of the treated group 'O' human cells for this test. The methods originally described by Burnet (1946) and subsequently modified by Aikawa and Meiklejohn (1949) and Florman and Kutch (1949) were all repeated at least twice with different pools of the "Enders" strain, but were found unsatisfactory. The cells so prepared were either auto-agglutinable at room temperature or at 37°C and did not conform to the "receptor gradient" described by Burnet et al. (1946).

The following method was used with a pool of "James" strain (from 3rd allantoic passage and with a H.A. titre of 1:320 using group 'O' human cells) and was found to satisfy the above two criteria. (Relevant results of titrations are shown in table VII, appendix (A)).

Steps: I. 2 ml. of a freshly harvested pool of the virus was mixed with 18 ml. of buffered (phosphate, p^H 7.2) saline and about 0.25 ml. of 'packed' group 'O' human cells were added to the diluted virus.

II. The mixture was incubated at 37°C in a water bath for 3 hours, with occasional shakings.

III/

III. The cells were washed in buffered saline for 3 times and on each occasion were resuspended to original volume.

IV. The resuspended cells after the third washing were further incubated for 1 hour. They were washed again as above and left overnight at 4°C.

V. The cells were washed once more and a 0·5% suspension prepared in the same diluent was incubated at 37°C for 1 hour.

The cells so prepared were found stable both at the room temperature and at 37°C and showed no tendency to auto-agglutinate. When tested against serial dilutions of the N.D.V., the influenza viruses (both PR 8 and Lee) and the James strain of the mumps virus the cells were agglutinated by the first two viruses only, Comparative titrations with untreated cells gave positive results with all the viruses.

It was observed in this connection that although virus could not be detected in the 3rd supernate of step (III), further incubation for 1 hour (step IV) resulted in the liberation of the adsorbed virus. The cells were auto-agglutinable both in phosphate-buffered and in calcium saline (as used for virus titrations) at all the stages, except after the overnight incubation at 4°C. Moreover, even after/

after repeated washings the cells did not conform to the receptor gradient until they were subjected to the cold temperature (step V).

On another occasion the cells were similarly prepared except that the initial period of contact was prolonged to 3½ hours, and after overnight preservation at 4°C were subjected to further in-:cubation at 37°C for 1 hour and washed in three changes of saline. The cell suspension (0·5%) was found satisfactory as before.

B. The serological test.

The method described by Burnet (1946) was followed. However, since the cells were found to be slightly unstable in calcium saline (at room temperature), only the buffered saline was used as diluent for the sera and the cells.

TREATMENT OF SERA WITH PERIODATE SOLUTION TO DESTROY THE NON-SPECIFIC INHIBITORS OF HAEMAGGLUTINATION BY THE MUMPS VIRUS.

Each of the sera was treated with periodate solution by the method described by Lündback (1949). The haemagglutination -inhibition (H.I.) tests were carried out by the method outlined in Chapter II.

0·2 ml. of undiluted serum was mixed with an equal volume of 0·05 M KIO_4 solution and the mixture was left at room temperature for 2 hours. Excess of the periodate was then neutralised by the addition of 0·8 ml. of 5% glucose solution. The serum was therefore diluted to 1 : 6 during this process. This was taken as the starting dilution of the serum for the H.I. test. The results have been expressed in term of the reciprocal of the final serum dilution after adding all the reagents.

It should be pointed out in this connection that the potassium or the sodium periodate powders (British Drug House) did not completely go into solution at the particular molar concentration recommended by Lündback. The solubility was not increased even by boiling for 10 to 15 minutes. Several serial dilutions of sodium periodate solution (M/100, M/250, M/500) also showed the undissolved salt. It was only when an M/1000 solution was prepared that the salt seemed to go into/

into solution completely.

Although 0·05 M KIO_4 solution was used for treating the human sera (Chapter VI , Section B), it was found that all the dilutions of the sodium salt, referred above, were effective in destroying the non-specific inhibitors in different pools of guinea pig sera. The result of one of the experiments is shown in tableIX, appendix (A).

It has also been observed that treatment of the serum with periodate alone results in a reduction of the non-specific inhibitors, which however is not complete unless glucose is also added. In fact, it seemed that glucose itself, in absence of any periodate, can cause a similar destruction of the non-specific inhibitors. On the other hand it was peculiar to find that increasing strengths of glucose solutions showed inhibition of the virus haemagglutination. The findings are shown in table VIII, appendix (A).

CHAPTER IV

SECTION (A): Haemagglutinins

SECTION (B): Concentration of the Virus

SECTION (C): Growth of the Virus in Embryonated Eggs

SECTION: (A)

HAEMAGGLUTININS OF THE MUMPS VIRUS

The recognition of haemagglutination by the mumps virus (Levens and Enders, 1945) was an early consequence of the development of methods of growing the virus in the amniotic and allantoic cavities of the chick embryo (Habel, 1945). From their study on the nature of the mumps virus, Beveridge and Lind (1946) concluded that the haemagglutinin is the virus particle itself, and that some of the complement fixing antigen is also contained in the virus particle. The haemagglutinating property is only exhibited by the virus cultivated in embryonated egg fluids; the virus grown inside the monkey parotid gland lacks in this property, although its presence is demonstrable by the complement fixation test (Levens and Enders, 1945; Enders and Levens, 1948; Henle et al., 1948a). Henle, Henle and Harris (1947) also observed that mumps infected chorio-allantoic membrane suspensions (soluble antigen) has no haemagglutinating property, but is rich in the content of the complement fixing antigen.

It has become customary to use the haemagglutination test (H.A. test) for the recognition and titration of the virus and for measuring the specific antibody against it. However, there are indications that the H.A. test does not always portray/

portray the true content of the virus, especially, the infective virus particle (Ginsberg et al., 1948a; Leymaster and Ward, 1948). Beveridge and Lind (1946) also suggested that the complement fixing and the haemagglutinating particles are not entirely identical.

The specificity of the H.A. test is considerably influenced by other factors. Firstly, various physico-chemical factors, such as, salt concentration, presence of "physiological" cations, etc. have been recognised by Magill and Sugg (1948). Secondly, the agglutination may be inhibited by non-specific factors present in egg fluids (Lanni and Beard, 1948; Beveridge and Lind (1946); Svedmyr, 1948; etc.) and in aqueous suspension of various tissues (Friedewald et al., 1947). Thirdly, other non-specific factors present in human and different species of animal sera are known to inhibit the haemagglutination of mumps - Newcastle disease - influenza group of viruses. These factors have been recently reviewed by Anderson (1949) and Burnet (1952) and will be discussed again in subsequent chapters.

It has been mentioned earlier that the mumps virus agglutinates red cells from fowls, guinea pigs and from human beings. Chu (1948) showed that the red cells from sheep are also agglutinated. While different haemagglutinating viruses are known to vary in their capacity to agglutinate the red cells from different species of/

of animals, little has been published so far to show the range of the types of red cells which are agglutinated by mumps virus. There is also no indication whether different strains of mumps virus vary in their capacity to agglutinate different types of red cells, except the one observation by Florman (1950). This worker reported that a strain of mumps virus (Habel strain) did not agglutinate group 'O' human cells as consistently as another strain (Enders strain).

As a preliminary to the investigations described in subsequent sections and chapters of this thesis, several experiments were performed to clarify some of the above observations. These will be presented in this section and the implication of the findings will be discussed later along with other evidence on the nature of the haemagglutinin.

EXPERIMENTAL AND RESULTS

A. Differences between the red blood cells from different fowls.

The agglutinability of the red cells from different fowls were examined by titrations against the Enders and the James strains of the mumps virus.

The cell suspensions (0·5%) were prepared from the red blood cells of different fowls by the method described previously. The strength of each suspension was checked by a haemocytometer count and the maximum variation between any two suspensions was within ten percent.

Doubling dilutions of the virus were prepared in bulk and distributed in equal volumes in a series of tubes, as for the haemagglutination test. The different cell suspensions were then added in series.

The results of titration of the Enders strain with six different cell suspensions are shown in table I. The titres varied over a range of eight - fold dilution. Corresponding results were obtained with the James strain and the cell suspensions, prepared under identical conditions, from 5 different fowls.

Similar observations have been made by Hirst (1943), Miller and Stanley (1944) in connection with their studies on the influenza virus, and by Beveridge and Lind (1946) on the haemagglutinin of mumps/

mumps virus. In view of the above discrepancy, the cells which showed the maximum titre were selected for any experiment during the present study. In a few cases where the fowl cells were pooled, only the selected birds were used for bleeding.

B. Reactivity of human red blood cells and those from different species of animals.

Several species of red cells were titrated against three different strains of the mumps virus to find out the range of cells that are agglutinable. The suspensions were prepared in normal saline from the cells packed in a centrifuge under identical conditions. The same diluent was used for preparing the virus dilutions. The cells were stored at 4°C for 0-4 days previous to their use in the test.

Each virus (from allantoic or amniotic fluid) was diluted in bulk and distributed in series. The red cells were added as in the previous experiment and the test was read after all the cells had settled. The results are shown in table II.

Similar titrations were made on several occassions with a few selected types of red cells. Some of these results are shown in tables III, IV and V.

It became apparent from these results that the red cells from fowls and guinea pigs give comparable titres/

titres for any virus preparation. Consistently positive results are obtained when the virus is titrated against the cells from different fowls or guinea pigs. In contrast, the group 'O' human cells and the cells from sheep and rabbits give lower titres and not infrequently indicate a negative reaction. The irregularity has been more apparent especially with the last two type of cells.

This discrepancy has not been further investigated. It is probable that apart from species specificity or individual variations various physico-chemical factors (Magill and Sugg, 1948) might be responsible. In this connection it may be mentioned that Florman (1950) has observed differences in the behaviour of different strains of mumps virus in haemagglutinating the fowl and group 'O' human cells.

Sheep cells treated with formalin and preserved at $4^{o}C$ for 3-4 days are still agglutinated, but the titres are usually lower than that obtained with fresh cells. Similar low titres were obtained when saponin treated lysed fowl cells (see chapter on electron microscopy), were used for titrations.

C. Effects of certain physical and chemical agents on the haemagglutinin of mumps virus.

The persistence of the haemagglutinin under conditions of storage, increase of temperature, formalin treatment/

treatment, irradiation with U.V. light and dialysis are shown in tables VI and VII. It was desirable to determine in advance these effects in connection with the preparation of the antigens and for other investigations described in the following chapters. Similar results were obtained when these factors were re-examined with different allantoic fluid pools of the same (Enders) strain or of a different strain (James).

Storage of the virus at -76°C preserved the H.A. titre for well over 6 months, although gradual reductions were evident after 1 to 2 months. Of the three strains, the Paul strain seemed to be unstable and the titre deteriorated rapidly in comparison with those of the others.

At -36°C the destruction of the haemagglutinin was still rapid and with different strains the property was lost within 3 to 6 weeks.

At 4°C the H.A. titre decreased rapidly although it could be demonstrated in some cases at the end of 4 weeks. With most preparations of undiluted virus, titres of less than 1:5 were obtained after 2 weeks. Moreover a considerable amount of urates was thrown down when allantoic fluid pools of the virus were stored undialysed at this temperature. As will be shown in next section, the haemagglutinin is lost in the precipitates of the urates.

Incubation of the virus at 37°C for a few hours has/

has a slight tendency to increase the H.A. titre (see also next section). However, some fall is noticeable after 3 to 4 days. It has been found to disappear completely after 7 days.

When the allantoic fluid virus was heated at 56°C the H.A. titre declined very rapidly. In several samples the minimum time at which the titre fell below 1:4 varied between 5-10 minutes. With diluted virus the time was still reduced.

Dialysis of the allantoic fluid or treatment with formalin do not lower the H.A. titre to any significant extent. In the latter case some reduction in the titre may be evident after storage at 4°C for 7-8 days; this is comparable to the result of storage even without formalin.

D. Nonspecific inhibition of the haemagglutinin:

(a) inhibitors in human and animal sera:

The presence of heat labile and heat-stable inhibitors in the sera of man and several species of animals were determined on a comparative basis. Different sera as shown in table VIII were inactivated at 62°C for 20 minutes except the human serum which was heated at 56°C for 30 minutes. All the sera were titrated as in H.I. (haemagglutination-inhibition) test described previously. A known convalescent rabbit antiserum against the homologous strain of virus/

virus used for the titration (Enders strain) was also included in the test.

It will be observed that the heat-inactivation did not lead to any significant diminution in the titre of the antibody. On the other hand the non-specific heat-labile inhibitors in different sera were considerably reduced (2 to 4 fold dilution).

The amount of the heat stable inhibitors varied in different sera. The inhibitors in the sera of rats, dogs, white Swiss mice and fowls were comparatively much less than those in the sera of rabbits, guinea pigs, brown mice and human beings. It is significant to note that the fowl sera contain a minimal amount of the non-specific inhibitors of the haemagglutinin. The results also indicate that fowls, rats and probably dogs should prove to be of obvious choice as experimental animals, specially when the antibody titrations are carried out by H.I. tests.

(b) Inhibitors in normal allantoic fluid:

The allantoic fluids from 3 to 5 embryonated eggs, belonging to different age groups (7 to 17 days), were pooled and stored at 4°C. Each pooled fluid was set up as for the H. inhibition test, so that, the serum was replaced by the allantoic fluid. All the dilutions were tested against 2 units of the virus (Enders strain). The fluids were tested from a minimum (final) dilution of 1:8.

Under the conditions of the test it was observed that/

that the allantoic fluids of 7 to 14 day old eggs did not exhibit any inhibitory effect on the haemagglutination. The fluids from 15 and 16 days old embryos showed slight inhibition at 1:16 and 1:32 respectively. The fluids at these ages of the embryos are usually viscid and show the presence of albuminous material. It is probable that the albumin was responsible for this inhibition.

The same titre was obtained when the fowl cells were added immediately after the virus and the allantoic fluids were mixed.

TABLE : I

Haemagglutination Test With Different Batches of Fowl Cell Suspensions and a Pool of Virus (Enders Strain)

Fowl no.	Final dilution of virus (r e c i p r o c a l)						
	160	320	640	1280	2560	5120	saline control
3326	4	4	4	4	±	0	0
2846	4	4	4	2	2	0	0
2502	4	4	1	0	0	0	0
3141	4	4	2	±	0	0	0
2886	4	1	0	0	0	0	0
2821	4	4	4	1	±	0	0

Degree of reaction : 4 = complete agglutination
0 = no . . . .

TABLE : II

Reactivity of Erythrocytes from Man and Different Species of Animals With Mumps Virus

Red cells from	ENDERS strain					JAMES strain						PAUL strain						Cell-saline contr.
	20	40	80	160	320	20	40	80	160	320	640	20	40	80	160	320	640	
Fowl(2)*	4	4	4	2	0	4	4	4	4	2	0	4	4	4	4	4	1	0
Gr."O"(2)	4	4	0	0	0	4	4	0	0	0	0	4	4	4	2	0	0	0
G.Pig(4)	4	4	4	4	±	4	4	4	4	1	0	4	4	4	4	2	0	0
Sheep(2)	4	4	1	0	0	4	4	4	1	0	0	4	4	2	0	0	0	0
Rabbit(2)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
White Rat (2)	4	4	1	0	0	4	4	4	4	1	0	4	2	0	0	0	0	0
..mouse(2)	0	0	0	0	0	9	±	0	0	0	0	0	0	0	0	0	0	0
Cat(1)	9	0	0	0	0	3	2	0	0	0	0	0	0	0	0	0	0	0
DOg(1)	4	4	4	4	0	4	4	4	4	9	0	4	4	3	0	0	0	0

H.A. test; titre in term of the reciprocal of the final dilution

ϕ c.f. results in table

* The figures in the brackets indicate number of animals from which the pooled suspension was prepared.

TABLE : III

Haemagglutination Test With a Pool of Mumps Infected Allantoic Fluid With Different Types of Red Cells

Cells	Dilution of the virus							Cell--saline control
	40	80	160	320	640	1280	2560	
Fowl(2)*	4	4	4	4	4	3	0	0
Group'O'(2)	4	4	3	2	0	0	0	0
G.Pig(3)	4	4	4	4	1	0	0	0
Rabbit(2)	3	4	3	2	0	0	0	0
Sheep(2)	4	4	4	4	0	0	0	0
Sheep(2) (formolated ψ)	4	4	3	0	0	0	0	0

0.5% cells in buffered normal saline

ψ 0.1% formalin

TABLE : IV

Haemagglutinin Content of Allantoic Fluids Tested With Different Types of Erythrocytes

Fluid no.	Fowl cells		Group 'O' cells		Sheep cells	
	1:20	1:40	1:20	1:40	1:20	1:40
1	4	3	2	0	4	4
2	4	4	4	4	4	4
3	4	4	4	4	4	2
4	4	4	0	0	0	0
5	4	4	4	2	4	0
6	4	2	4	4	4	4
7	4	4	4	0	0	0
8	4	4	4	1	4	2
9	4	4	2	0	4	1
10	4	4	4	4	4	4
11	4	4	4	2	2	0
12	4	4	0	0	4	0

Each fluid tested at dilutions (final) of 1:20 & 1:40 with 0.5% of appropriate cell suspension

TABLE : V

Haemagglutination Titration of Mumps Virus(Enders Strain) from Eggs Inoculated With Different Doses of the Virus

Cells	Eggs inoculated with different dilution of seed virus									
	10^{-3}	10^{-4}			$10^{-4.5}$			10^{-5}		
	1	1	2	3	1	2	3	1	2	3
Fowl	4	4	4	4	4	4	4	4	4	4
Sheep	4	4	4	4	4	4	4	4	4	4
G.pig	4	4	4	4	4	4	4	4	4	4
Gr.'O'(a)	3	2	4	1	1	1	3	4	4	2
... (b)	2	2	4	0	1	1	1	4	4	2

All fluid tested at a final dilution of 1:40

TABLE : VI

H.A. titration of a pool of mumps (Enders strain) infected allantoic fluid after treatment with physical and chemical agents

Treatment	H.A. titration (reciprocal of final dilution) 10	20	40	80	160	320	640
Original fluid	4	4	4	4	2	00	0
4°C for 1 week(s)	4	4	4	3	0	0	0
.. .. 2 ..	4	4	2	0	0	0	0
.. .. 4 ..	3	0	0	0	0	0	0
R.T. for 3 days	4	4	2	±	0	0	0
... .. 7 ..	4	1	0	0	0	0	0
37°C for 1/2hr.	4	4	4	4	3	1	0
.. ..1 1/2 hrs	4	4	4	4	4	2	9
.. .. 3 ..	4	4	4	4	4	2	0
.. .. 24 ..	4	4	4	4	2	9	0
.. .. 4 days	4	4	3	0	0	0	0
56°C for 5 mins.	4	2	0	0	0	0	0
.. ..10 ..	1	0	0	0	0	-	-
.. ..15 ..	0	0	0	0	0	-	-
Irradiated: 1/2 hr. for	4	4	4	4	4	±	0
3hrs.	4	4	4	4	4	1	0
Formalin(0.1%) for 8 days at 4°C	4	4	4	4	±	0	0
Dialysis at 4°C for 48 hrs.	4	4	4	3	3	±	0

TABLE : VII

The decline in titre of the haemagglutinin after storage at -36°C and -76°C

Virus	Pool	H.A. titre before storage	Time of storage	H.A. titre after storage at -36°C	at - 76°C
Enders	1	1:320	1 month(s)	1:80	-
		"	3 "	-	1:320
	2	1:1280	6 "	-	1:320
	3	1:160	1/2 "	1:160	-
			1 "	1:80	-
			2 "	1:10	
James	1	1:320	5 "	-	1:80
Paul	1	1:320	1 "	1:10	-

TABLE : VIII

Haemagglutination-Inhibition Test With Unheated and Heated Sera From 6 'Normal' Animals and Man

Serum	Unheated or Heated	Final dilution of the serum (reciprocal) 8	16	32	64	128	256	512	1024	2048	4096
Rat (4)	Un.	0	0	1	3	4	4	4			
	Ht.	2	3	4	4	4	4	4			
Rabbit (4)	Un.	0	6	0	0	0	1	4			
	Ht.	0	0	0	2	4	4	4			
Dog (2)	Un.	0	1	1	2	4	4	4			
	Ht.	1	2	2	4	4	4	4			
G.Pig. (6)	Un.	0	0	0	0	1	3	4			
	Ht.	0	0	1	2	4	4	4			
Mouse (brown) (12)	Un	0	0	0	0	0	2	4			
	Ht.	0	0	3	3	3	4	4			
Mouse (white) (12)	Un.	0	0	0	0	2	3	4			
	Ht.	1	1	3	3	4	4	4			
Fowl (2)	Un.	1	3	4	4	4	4	4			
	Ht.	3	4	4	4	4	4	4			
Man	Un.	0	0	0	0	0	2	4			
	Ht.	0	0	0	1	3	4	4			
Mumps anti-sera (rabbit)	Un.	0	0	0	0	0	0	0	0	0	4
	Ht.	0	0	0	0	0	0	0	0	2	4

All unheated sera were preserved frozen after collection
The human serum was heated at 56°C for 1/2 hour; others were heated at 62°C for 20 minutes.

Figures in brackets indicate the number of sera pooled.

THE CONCENTRATION OF MUMPS VIRUS

To improve the immunising potency of influenza vaccines a variety of methods have been developed for the concentration and purification of influenza virus. These techniques utilise various physical and chemical methods and have been well reviewed by Anderson (1946), Pirie (1946), van Rooyen and Rhodes (1948). Cox et al. (1947) reported on the efficacy of methyl alcohol in the concentration of influenza virus.

Recently, Forster and Carson (1949) and Pollard et al. (1949) have extended some of these techniques in order to concentrate the mumps virus. The first two workers employed the methyl alcohol precipitation method and the red cell adsorption and elution technique to concentrate the virus. Pollard et al. attempted to study the efficacy of the methyl alcohol for the same purpose.

During the course of the present study several experiments were performed to concentrate the mumps virus for

(i) use as diagnostic antigens, and

(ii) to obtain suitable material for the examinations by electron microscopy.

The results of these experiments and the pertinent observations will be described in this section.

(A) Adsorption of the mumps virus on urate deposit

It was often noticed that pooled allantoic fluids from embrys older than 13 or 14 days contained white streaky deposits of urates. Even when the pooled/

pooled fluid was apparently clear after the harvest, the urates would appear in the fluid when the latter was frozen at -36°C and thawed slowly at room temperature. The deposit was usually granular in nature. Similar heavy precipitates of the urates were obtained when the allantoic fluids were stored at 4°C. Amniotic fluid did not usually show the the deposit unless it was mixed with the allantoic fluid during collection.

It was found that a pool of mumps infected allantoic fluid, containing the urate deposit, lost a great deal of the haemagglutinin when the deposit was removed by centrifugation. To explain this loss, the following experiments were undertaken.

Experiment I

An allantoic fluid pool, containing the Enders strain of the virus, was repeatedly frozen and thawed over a period of 2 days. A mixture of granular and flocculent deposits was obtained.

A portion of the fluid (10 ml.) was centrifugated at 2,000 r.p.m. for 5 mins. and 9/10th of the supernatant fluid was removed. The fluid overlying the deposit (about 0·8 ml.) was discarded. The deposit was resuspended in phosphate buffered normal saline up to original volume and placed in a water bath at 37°C for 1 hour. This fluid was shaken from time to time in order to break down the precipitate as far as possible. At the end of the period it has centrifugated as above and the upper 9/10th/

9/10th of the supernate was collected again. The deposit was resuspended in saline to 1/10th of the original volume and incubated at 37°C for a further period of 1 hour.

The different fractions, along with the original fluid, were titrated for the haemagglutinin content. After recording the results each tube was thoroughly shaken to resuspend the cells. The test was read again after further incubation at 37°C for 1 hour. These results are shown in table I.

It will be evident from this experiment that considerable amounts of the virus are adsorbed on the urate deposit. If the deposit is removed or if the pooled fluid is not thoroughly shaken, a large amount of the available haemagglutinin may be lost. When the deposit is resuspended to original volume, the titre of the fluid reaches practically to the same level as that of the original fluid.

The results after incubation of the test at 37°C show that at this higher temperature of the incubation the original pooled fluid and the supernatant I, exhibit higher titres. In contrast, the supernatant II and the final deposit do not show this tendency. It appears probable that at 37°C the virus aggregates are inclined to be broken up, so that, more virus particles are available for the haemagglutination reaction. In addition, some of the virus elementary bodies are also eluted from their adsorbed state. This latter assumption probably explains the release of the virus into the supernatant/

supernatant (II) fluid even after its loss in supernatant I.

EXPERIMENT II

This experiment, which is similar to above, was carried out with another pool of the same virus strain. The first deposit was resuspended to 1/10th the original volume and incubated at 37°C for 30 minutes. A drop of the fluid was removed for the haemagglutination (H.A.) test, and the rest was incubated further until 90 minutes.

Different fractions as shown in table II were tested by the H.A. test. A part of the original fluid which was dialysed earlier, was also included in the titration.

It will be observed that there was a concentration of 20 to 30 fold when the deposit was resuspended to 1/10th the original volume. Apart from the concentration factor, it is worth noting that all the components of the allantoic fluid except the virus and the urates are eliminated.

Since the urates may be removed by further centrifugation and dialysis, it seems possible to obtain a comparatively pure preparation of the virus by this simple procedure. It must be admitted, however, that some of the virus is liable to be lost in the supernatant allantoic fluid

(B)/

(B) Methyl alcohol precipitation

For the concentration of mumps virus by methanol precipitation the procedure outlined by Cox et al. (1947) was followed with some modifications. The following experiments were performed.

Experiment I

To a pool of mumps infected allantoic fluid (Enders Strain) enough methyl alcohol (analytical reagent) was added to make a 20% solution. Both the allantoic fluid and the methyl alcohol were chilled beforehand at about 1°C and -10°C respectively. The mixture was incubated at -10°C (ice-salt mixture) for 2½ hours, so that there was a maximum precipitation of the virus with as little denaturation as possible. The precipitate (I) was then sedimented in an angle-head centrifuge (2,500 r.p.m. for 30 mins.) at 4°C and resuspended in buffered (phosphate) saline pH 7·2. The volume of the saline was 1/10th of that of the allantoic fluid.

Table III(a) shows the haemagglutinin content of different fractions. It will be seen that 60 to 70% of the virus was recovered from the deposit.

It was observed that the precipitated material tended to go into solution whenever there was an increase in the temperature of the mixture. Since there were some technical difficulties in running the centrifuge at -10°C, it was felt that the difference in the temperature was responsible for the 30 to 40% loss of the virus in the supernatant.

To/

To recover the lost virus, the supernatant was subjected to the following treatment. It was placed inside a centrifuge tube, and frozen at -36°C. The metal bucket of the centrifuge was similarly chilled. The frozen material was then centrifugated for 5-10 minutes at 4°C. During spinning, there was a gradual thawing of the mixture. Any precipitate that was formed was quickly deposited at the bottom of the centrifuge tube. Very little of the precipitate went into the thawed supernatant. The deposit was resuspended into 1/10th the original volume and the process was repeated several times till the supernatant did not show the presence of any virus.

It will be observed from table III(a)that the whole of the virus could be recovered from the original supernatant fluid (I) by the latter method.

Experiment II

A pool of dialysed allantoic fluid infected with Enders strain (recently prepared 'viral' complement fixing antigen) was mixed with one-fifth its volume of methyl alcohol as in experiment I.

The mixture, contained in a centrifuge tube (conical bottom) was held at -10°C for 30 minutes and then quickly frozen at -36°C. The tube was placed inside a chilled (-36°C) metal bucket and spun in an angle head centrifuge at room temperature (4,500/

(4,500 r.p.m. for 5 to 6 minutes). The precipitate (I) was quite heavy, and was resuspended in buffered saline to 1/10th of the original volume. The same cycle was repeated with the supernatant and a second precipitate (II) was collected similarly.

The precipitates went into solution with a little shaking and after a brief period of 5-10 minutes at 37°C in a water bath. The different fractions as shown in table III(b) were tested for the virus content by H.A. and complement fixation tests.

The H.A. titrations were performed as before. The C.F. antigen was determined by titrations against a known convalescent human serum at a fixed dilution of 1:8. A normal serum (pooled) was also used at 1:4 dilution as control; it did not contain any mumps C.F. antibody even at 1:2 dilution.

The results are shown in table III(b). It became apparent that practically the whole of the haemagglutinin can be recovered by two cycles of the methanol precipitation method. The yield of the virus, in terms of the haemagglutinin, appeared to exceed 100% - if the relative titres of the original fluid and of the ten-fold concentrates are considered together. A similar phenomenon has also been noted by Cox et al. (1947) with influenza viruses and Forster and Carson (1949) with the mumps virus (c.f. adsorption of the virus on urates).

The effect of methanol on the complement fixing/

fixing antigen of mumps virus is somewhat similar to that on the haemagglutinin. The precipitate did not show any anticomplementary property. There was no fixation with the normal serum.

It is however noteworthy that in spite of the concentration of the C.F. antigen, the total yield was not of the same order as that of the haemagglutinin. Some of the antigen was apparently lost because the titre of the supernatant alcohol - allantoic fluid mixture cannot account for the discrepancy. The significance of this finding will be discussed later.

Experiment III

This experiment was undertaken to examine the nature of the methanol precipitated components of the allantoic fluid. A pool of allantoic fluid from 14 day old uninoculated eggs was treated with methyl alcohol by the same method as used in experiment II and the cycle was repeated 4 times.

It was observed that the 1st. cycle precipitate was considerably more heavy than that in experiment II and did not easily go into solution. In subsequent cycles the precipitates gradually became lighter and flaky in nature, and readily went into the solution. There was practically no deposit at the end of the last cycle.

This simple experiment serves to point out that during the methanol precipitation some amount of the nitrogenous/

nitrogenous elements (proteins) of the allantoic fluid is also thrown down along with the virus.

Experiment IV

The methanol precipitated virus (the 1st. and the 2nd. cycle deposits) from experiment II was inoculated into the allantoic cavity of two batches of 8 day old eggs. The virus was diluted at 10^{-1} and each egg received 0·1 ml. All the eggs died on the second or the third day after inoculation. The inoculated fluids were found to be bacteriologically sterile.

In view of the results from experiment III, it appears probable that the death of the embryos was due to the toxicity and high concentration of the nitrogenous components of the allantoic fluid.

(C) Centrifugation of the Mumps Virus

Henle et al. (1947) showed that the mumps virus could be deposited from infected allantoic or amniotic fluids at 20,000 r.p.m. for 20 minutes. According to filtration experiments by Habel (1945) and others it appeared that the virus is of a size which could also be thrown down at 15,000 r. p.m. Several experiments were made to determine whether the virus is deposited at this speed.

That the method has proved satisfactory will be evident/

evident from the results described in detail in the chapter on electron microscopy. Additional evidence has been presented in the section on preparation of 'soluble' complement fixing antigen and its standardisation for diagnostic work. To show the effectiveness of this method in concentrating the mumps virus two controlled experiments will be described in this section.

Experiment I

A pool of dialysed infected allantoic fluid (James strain) was clarified by a low spin at 2,500 r.p.m. for 5 minutes to throw down gross particles. A measured amount of the fluid was then spun at 15,000 r.p.m. in a bucket type (Ecco) centrifuge for 1 hour. Equal amounts of the fluid were also spun for 2 hours. The centrifugation was carried out at room temperature. The upper and lower portions of the supernatant fluid from each tube were removed as shown in table IV. Each of the deposits was resuspended in buffered normal saline to 1/20th the original volume. Different fractions along with the sample of the original fluid were titrated by haemagglutination and by complement fixation tests.

The results indicate that the bulk of the agglutinin and of the complement fixing antigen were removed at the end of 1 hour. The upper portion of the supernatant fluid showed very little of/

of the virus when compared with the resuspended deposit. Apparently, a better concentration was achieved by prolonging the period of centrifugation.

From this and other similar experiments it was found that for practical purposes a highly concentrated preparation of the virus could be obtained by centrifugation for 1 to 1¼ hours. The small amount of the virus that remains in the supernatant must have come as a result of the convection current of the fluid consequent to the room temperature.

These experiments also indicate that the movement of the complement fixing antigen under the centrifugal force although parallel to that of the haemagglutinin is not identical. Comparatively, there is less difference between the titres of the supernatant and the original fluid by complement fixation test. Possibly some component of the mumps virus has the complement fixing property and does not cause haemagglutination. It is not possible to say whether it represents the 'soluble' component of the complement fixing antigen released into the allantoic fluid.

The/

The following results obtained during the preparation of soluble complement fixing antigens also illustrate the effectiveness of the centrifugation method.

Fraction	H.A. titre	
	Enders Strain	James Strain
1. Last washing of the C.A. membranes	1:2	1:2
2. Supernatant from 20% membrane suspension after 2,500 r.p.m. for 20 minutes	1:20	1:35
3. Above supernatant after 15,000 r.p.m. for 1 hour ...	<1:5	<1:10
4. Deposit of high speed centrifugation resuspended to 1/5th original volume of 3 (above)....	1:480	1:640

The fractions No. 2 and 3 (James Strain) were titrated for the content of the complement fixing antigen. Both showed a titre of 1:40. This complement fixing antigen in the C.A. membrane suspension is same as the 'soluble' antigen described by Henle and her co-workers. The above results show that it is not thrown down at 15,000 r.p.m., it behaves differently from the haemagglutinin and the 'viral' complement fixing antigen which are concentrated to a considerable extent at this speed of centrifugation. The same effect was observed in the results of another test.

For the usual clarification of a pool of allantoic or amniotic fluid, a period of centrifugation (in angle-head) for 5-10 minutes at 2,500 r.p.m. has been found to be satisfactory. No difference in the H.A. titres/

titres of the upper and lower portions of the supernatant fluid could be observed under such condition. An experiment was performed to find out whether there is any loss of virus in the supernatant clarified fluid when the period of centrifugation was prolonged.

Table V shows the result of centrifugation at 4,000 in an ordinary 'bucket' type centrifuge for 1 hour. It will be observed that there is no significant difference in the titres of the supernatant and the original fluid. The deposit however shows some concentration. Apart from the fact that it was resuspended in a small volume of fluid, it is possible that some of the virus aggregate entangled in cellular deberis was responsible for the higher titre.

(D) Concentration of the mumps virus by adsorption and elution from erythrocytes.

It has been shown in the earlier section that the virus will agglutinate red cells of different species of animals and of human beings. The red cells of fowls and guinea pigs are agglutinated with regularity; the results with group 'O' human and sheep cells are sometimes unpredictable.

With selected batches of sheep, group 'O' and fowl cells several experiments were performed to determine their usefulness for the concentration of the virus. Certain discrepancies in the behaviour of the haemagglutinin and the complement fixing antigens/

antigens of mumps virus were revealed by the application of the adsorption and elution techniques. In addition, a few experiments with laked (saponin treated) fowl erythrocytes were undertaken as a preliminary to the preparation of specimens for electron microscopy of the virus.

(I) Adsorption on and elution from 2% erythrocyte suspension:

Washed, packed and measured amounts of fowl, group 'O' and sheep red cells were added to separate fractions of pooled infected (Enders strain) allantoic fluid. The final concentration of the cells was 2% in all cases. The mixture was then chilled for 2 to 3 hours at a temperature of 0° to 2°C (salt-ice bath) for adsorption of the virus. The cells were spun down in chilled centrifuge buckets at 3,000 r.p.m. for 2 minutes and resuspended in buffered normal saline ($p^H 7 \cdot 2$) to 1/10th the original volume of the allantoic fluid. The virus was allowed to elute at 37°C (water-bath) for another 2 to 3 hours. The H.A. titres of the original fluid, the supernatant and the eluate were then determined with 0·5% fowl cell suspension.

Table IX shows the results of some of these experiments. It will be observed that under identical conditions, sheep cell suspensions showed better adsorption of the virus than the fowl cells. The use of group 'O' cells gave a poor result. In spite of weaker haemagglutination reactions, the sheep/

sheep cells appear to adsorb more virus particles and to release them more completely.

An attempt was made to determine the minimum time for adsorption on 2% fowl cell suspension. The procedure followed was the same as described above. Table VI shows the result of this experiment. It shows that there is a quick fall in the titre of the supernatant fluid within 15 minutes. Probably very little of the virus is adsorbed after 30 minutes, and a state of equilibrium is maintained even up to 6 hours.

In other experiments of a similar nature it was found that slight dilution of the pooled infected allantoic fluid (1:5 or 1:10) in buffered saline before the addition of cells showed better adsorption of the virus.

Still better results were obtained when centrifugated virus, resuspended in saline, was used for adsorption. These indicate that the adsorption is influenced by the factors present in allantoic fluid and they are possibly the same as the inhibitors of haemagglutination.

The effect of temperature on the adsorption of mumps virus was also determined (table VII). It was found that even at room temperature a great deal of the virus remains adsorbed on the cells. It elutes when the mixture is left at room temperature and probably the process is gradual and uniform. At 37°C there is apparently no adsorption of the virus./

virus. It is probable that the virus which is adsorbed elutes quickly. In the course of another experiment it was observed that even after a prolonged contact for about 3 hours when the cells were washed with cold saline and then allowed to liberate the virus, a significant amount of the haemagglutinin was recovered. The slightly higher titre of the supernatant fluids at 37°C, in spite of the adsorption, is probably due to the release of more virus particles from their aggregated state at this temperature.

(II) Adsorption of the virus on 'laked' fowl cells.

The method of preparation of the laked cells by saponin treatment is given in detail in the chapter on electron microscopical examinations. The results of an experiment carried out under identical condition as above with the intact fowls cells are shown in tables X and XI. It showed that the results are closely similar. However, it was observed that (i) the laked cells do not adsorb the virus so completely as the intact cells, (ii) the adsorption is probably maximum at about 2 hours after the cold incubation and (iii) there was some tendency of the virus to elute after 4 hours, even at 0°C.

Another peculiarity was noticed during the use of the laked cells. The agglutinated cells formed into stronger clumps and were not so easily resuspended on shaking. This was particularly marked/

marked with the purified and concentrated virus.

(III) Adsorption on and elution from heavier suspensions of the red cells.

(a) Fowl cells: Two, five and ten percent fowl cells were used for adsorbing the virus at 0°C for 2½ hours. The virus was recovered after elution at 37°C for 3 hours. The results of comparative H.A. titrations (table VIII) showed that there was better adsorption and recovery of the virus with increased density of the red cell suspension.

(b) Sheep cells:

Because of the promising results of adsorption and elution of the virus with sheep cells as mentioned earlier (table IX) and the indication that heavier suspensions might be useful, the following experiments were performed.

Experiment I

In to different tubes containing equal volumes of undiluted virus (Enders strain), varying amounts of packed sheep cells were added to make final concentrations of 5, 10, 15, 20 and 30 per cent. The tubes were placed in a bath (salt-ice) at ±1°C and left overnight inside a frigidaire. Another set of mixtures was similarly prepared consisting of 5,10 and 20% red cells. This was incubated at ±1°C for 1 hour.

After the period of incubation in cold, each mixture/

mixture was spun quickly at 2,500 r.p.m. for 5 minutes at 4°C. The supernatant fluid was removed as completely as possible and the deposited cells were resuspended in normal saline (buffered at $p^{H}7{\cdot}2$). The virus was allowed to elute at 37°C for 3 hours. At the end, the eluate was collected by centrifugation at room temperature and all the fractions were examined for the contents of the haemagglutinin and the complement fixing antigen.

The H.A. and the C.F. tests were performed on different days and the fluids were stored at -36°C during this interval. A pool of high-titre homologous antiserum from rabbits was used for the latter test. The fluids were only tested for the anticomplementary property. No normal serum was used as control because of insufficient amounts.

It was observed that with 20% and 30% cells there was slight haemolysis during the elution. This was particularly true when the virus-cell mixtures were left for overnight incubation in the cold. The results of the titrations are shown in tables XII (A) and (B).

As would be expected, a better result was obtained when the adsorption took place for the shorter period of 1 hour. No haemagglutinin was detected in the supernatant fluid even at 1:10 dilution of the virus (original titre 1:160). Whereas the virus was adsorbed at every density tested, there was a definite indication that increased con-

centration/

concentration of the cells resulted in a greater yield of the haemagglutinin in the eluate. Twenty and thirty percent cell suspensions seemed to be most effective for this purpose.

The results of this experiment also manifested certain peculiarities of the nature of the haemagglutinin and the complement fixing antigen. It supported the earlier observations made in connection with the centrifugation analysis. It will be observed from the tables that the complement fixing antigen is not adsorbed so effectively as the haemagglutinin. The fold-reduction in titre is comparatively less. The eluate does not show a high level of the antigen, unless a higher density of the cells is used for adsorption. Moreover, the released virus does not show a definite fixation and there is a tendency to zoning. Some of these facts will again become apparent in subsequent experiments. The significance of these findings will be discussed later.

Experiment II

Two allantoic fluid pools containing the James and the Enders strains of the virus were each diluted at 1:5 with buffered normal saline. Enough packed sheep cells were added to make 20% suspension. Each mixture was distributed in equal amounts in three tubes and they were placed in the salt-ice bath at $\pm 1^{\circ}$C. At 20, 40 and 60 minutes one of the tubes from each series was centrifuged for the collection/

collection of the supernatant fluid.

All the fractions together with the original fluids of either strains were tested for the H.A. titre. It was found that there was a fall in titre from 1:640 to less than 1:10 at 20 minutes in both the cases. The 40 and 60 minutes samples also showed the same titre of 1:10 - the minimum dilution used during the test.

The rapid and effective adsorption of the virus was possibly due to the use of a higher percentage of the cells and the slight dilution of the original virus pool (c.f. results of the experiment below).

(IV) Adsorption and elution of the haemagglutinin and the complement fixing antigen from sheep cells.

(A) Two experiments were undertaken to determine the optimum times for the adsorption and the elution from sheep cells - using a 20% concentration of the cells.

(1) The first experiment was performed with a dialysed 'Viral' antigen (James strain). The cells were added to the undiluted antigen. The adsorption was allowed to proceed for two and a half hours and samples were removed at intervals to collect the supernatant adsorbed antigen. Finally, the remaining cells with adsorbed virus were spun down and then washed in two changes of cold saline. They/

They were resuspended in buffered saline to the original volume (the volume before centrifugation was started). All manipulations, including the centrifugation, were carried out at a temperature of about 4°C.

The resuspended cells were distributed in equal volumes into several smaller tubes. They were placed in water bath at 37°C to elute the virus. Samples were again collected at intervals and the eluted virus was separated from the cells by centrifugation. The different fractions were titrated by H.A. and C. Fixation tests as in experiment (III (b) Expt. I) described earlier. The results have been shown in table XIII.

It will be seen that the maximum adsorption of the haemagglutinin did not occur until at least 1 hour. This is probably due to the use of undiluted allantoic fluid. The adsorption was practically complete at 1½ hours when the titre was reduced from 1:640 to less than 1:5. The elution was comparatively rapid and the whole of the haemagglutinin was recovered within 2 hours. It appears that the elution probably occurs in steps and is analogous to the phenomenon which has been described in connection with the elution of the influenza viruses. An increase of the period of elution beyond 3 hours does not yield any additional amount of the haemagglutinin.

The/

The result of the C.F. test supports the findings of the earlier experiment. In fact, the discrepancy is more marked in this case. It will be interesting to note that:

(i) at the end of 2½ hours of the adsorption period, there was only a two fold reduction in the titre of the C.F. antigen (viral);

(ii) during the washing of the cells, the lowest dilution of the test showed incomplete fixation of the complement although the complement control tubes were satisfactory;

(iii) there was a release of the complement fixing antigen in the eluates to high titres and it was somewhat parallel to that of the haemagglutinin, and

(iv) most of the eluates showed marked zoning.

(2) The second experiment was performed with a sample of 'soluble' complement fixing antigen (prepared from James strain). It was adsorbed with 20% sheep cells under identical conditions. As shown in table XIV, different fractions were collected and tests were made similar to those in the first experiment.

The results show that small amounts of the haemagglutinin which may escape from being deposited by the high speed centrifugation (15,00 r.p.m. for 1 hour) are readily adsorbed by the sheep cells. It/

It is therefore possible to free any trace of the haemagglutinin from the 'soluble' complement fixing antigen by this method. The adsorbed haemagglutinin can be recovered by elution at 37°C for 3 hours.

The C.F. titre of the soluble antigen is only slightly reduced after the adsorption. It is of interest to find that some of the adsorbed antigen is readily dissociated when the cells are washed. In contrast, no haemagglutinins are released during the process. The release of soluble antigen is quicker than that of the haemagglutinin, although at the end of 3 hours the eluate shows maximum titres of both these components. The zoning which was noted with the eluates containing the viral antigen is more marked in this case and even at the lowest dilutions the fixation of the complement appeared to be incomplete.

(B). In a similar experiment as the two described above, 5% sheep cells were used for adsorption. The eluates were made in smaller volumes, so that concentrated specimens could be obtained. After the first eluate was obtained at a p^H of 7·2, the cells were spun down and resuspended in fresh saline at p^H7·8. Each of the process lasted for 3 hours. The results are shown in table XV and have been discussed below along with those of the next experiment.

(C). Different preparations of viral and soluble/

soluble antigens, including crude suspensions of C.A. membranes before high speed centrifugation, were adsorbed with 5% (fresh) sheep cells. The eluates were made into original volume. Each of the antigens was also adsorbed with 5% formolated sheep cells (treated before hand with 0·1% formalin) under identical conditions. No eluates were collected from these cells. The titration of different fractions by H.A. and C.F. tests are shown in table XVI.

The results of these two experiments are consistent with those described earlier. They show that the haemagglutinins were not completely adsorbed, although most of the adsorbed haemagglutinins could be recovered from the eluates. It may also be observed that unless the eluate is concentrated the C.F. antigen may not be detected in significant amount. This is particularly true in case of the soluble C.F. antigen.

The variation in the p^H of the eluting saline seems to have some effect on the yield of the virus. Apparently, the C.F. antigens (both viral and soluble) are released fairly completely at $p^H 7·2$. In contrast, a considerable amount of the haemagglutinin is still adsorbed on the cells and this elutes at $p^H 7·8$. This latter finding supports an earlier observation by Pollard et al. (1949) that mumps harmagglutinins elute better at an approximate p^H of 8·0.

The/

The differential release of the C.F. antigens and the haemagglutinin is possibly due to their distinctive character. The results also suggest that the optimal p^H for the elution of the C.F. antigens may be in the region of 7·2. There may be an alternative explanation. It is probable that the component of the mumps virus which shows only the C.F. property is adsorbed onto the cells in a loose fashion, quite distinct from that of the haemagglutinin, and is readily released when the temperature is raised from 0°C to 37°C.

It has been shown previously that formalin-treated sheep cells may be agglutinated by the mumps virus, although not to an extent comparable with fresh cells. For adsorption purposes therefore their use seems to be limited. No significant change in titres of H.A. and C.F. tests could be observed with any of the treated antigens.

DISCUSSION /

TABLE : I

Adsorption of Mumps Virus on Urates Present in the Allantoic Fluid

Experiment :I

Specimen	Reading 1st.:R.T. 2nd.:37°C	Haemagglutination test (reciprocal of final dilution) 80	160	320	640	1280	2560	5120
Untreated fluid—containing the urates	1st.	4	4	4	4	2	0	0
	2nd.	4	4	4	4	4	2	0
Supernant.I after first centrifugation	1st.	4	4	2	1	0	0	0
	2nd.	4	4	4	4	2	0	0
Supernant. II after resuspending the deposit to original volume	1st.	4	4	4	4	2	0	0
	2nd.	4	4	4	4	4	±	0
Last deposit in 1ml.of saline	1st.	4	2	0	0	0	0	0
	2nd.	4	3	±	0	0	0	0

TABLE : II (as above)

Experiment :II

Specimen	H.A.titre (reciprocal) 100	200	666	1000	2000	3000
1. Pooled allantoic fluid (undialysed)	4	4	3	0	0	0
2. .. ..(dialysed)	4	4	4	±	0	0
3. Supernatant after centrifugation	2	0	0	0	-	-
4. Resuspended deposit in 1/10th.original volume + 37°C for 30 mins.	4	4	4	4	2	0
5. Above + 37°C for 90 mins.	4	4	4	4	4	2

TABLE : III a,b.

Concentration of Mumps Virus by Methyl Alcohol Precipitation

(a) Experiment : I

Specimen	Haemagglutination titre							Calculated end-point
	1/2	1/10	1/100	1/250	1/500	1/1000	1/2000	
Allantoic fl. (pool)	4	4	2	0	0	0	0	1/100
Precipitate I	4	4	4	4	4	0	0	1/750
.. .. II	4	4	2	0	0	0	0	1/100
.. .. III	4	4	4	4	3	1	0	1/625
.. .. IV	4	2	0	0	0	0	0	1/10
Last supernant	0	0	0	0				<1/2

(b) Experiment : II

Specimen	Haemagglutination titre(reciprocal)									Calculated endpt.	Titre of Com.Fix. antig.
	2.5	10	50	100	250	500	1000	2000	3000		
Dialysed allantoic fluid	-	-	4	4	2	0	0	0	0	250	1/32
Precipitate I (conc.X10)	-	-	-	4	4	4	4	4	0	2500	1/128
PrecipitateII (conc.X10)	-	-	4	4	2	0	0	0	0	250	1/16
Supernant fld. after ppt.II	0	0	0	0	-	-	-	-	-	<2.5	<1/2

4 =complete agglutination

TABLE : IV

Centrifugation of Mumps Virus(James strain) at 15,000 r.p.m.

Fraction	Time (hr.)	H.A. test								Comp-fixation test							Com. con. 1:5
		40	80	160	320	640	1280	2560	5120	10	20	40	80	160	320	640	
Original fluid (dialysed)	0	4	4	4	4	0	0	0	0	0	0	2	4	4	4	4	4
Upper9/10th. (supernatant fl.)	1	4	2	0	0	0	0	-	-	0	1	3	4	4	4	-	4
Lower1/10th. (sup. fluid)	(1)	4	4	3	0	0	0	-	-	0	0	2	4	4	4	-	4
Deposit ϕ		4	4	4	4	4	4	4	3	0	0	0	0	0	0	4	4
Upper4/5th. (supernatant fl.)		2	0	0	0	0	0	-	-	0	3	4	4	4	-	-	4
Lower1/5th. (sup. fluid)	(2)	4	4	2	0	0	0	-	-	0	2	4	4	4	-	-	4
Deposit ϕ		4	4	4	4	4	4	4	2*	0	0	0	0	0	2*	4	4

H.A.test: reciprocal of the final dil./ C.F.test: rec.of initial dil.

Slightly lower titre in comparison with that of the 1 hour deposit was due to the fact that a portion of the fluid was removed by an electron microscope grid placed at the bottom of the centrifuge tube.

ϕ Deposit resuspended in 1/20 th. of the original volume.

In complement fixation test: 0=complete fixation
4= .... haemolysis

TABLE : V

Centrifugation of Mumps Virus in an Ordinary Centrifuge at 4,000 r.p.m.

Fraction	H.A. test							C.F.test							Compl. contr. (1:5)
	80	160	320	640	1280	2560	5120	10	20	40	80	160	320	640	
Orig.fluid.	4	4	4	0	0	0	0	0	0	2	4	4	4	4	4
Supernat. (upper5/6th)	4	4	4	0	0	0	0	0	0	2	4	4	4	4	4
Supernat. (lower1/6th)	4	4	4	3	0	0	0	0	0	2	2	4	4	4	4
Deposit*	4	4	4	4	4	3	0	0	0	0	0	0	2	4	4

* Deposit resuspended in 1/20 th. of the original volume.
Explanatory notes as in table above

Table : VI

Effect of Time on the Adsorption of Mumps Virus(Enders Strain) with Intact Fowl Cells at a Temperature of 0°C

Period of adsorption	H.A. test with the supernatant fluid							
	40*	80	160	320	640	1280	2560	5120
0 hour	4	4	4	4	4	4	2	0
1/4 ..	4	4	4	3	0	0	0	0
1/2 ..	4	4	2	0	0	0	-	-
1 ..	4	4	2	0	0	0	-	-
2 ..	4	4	1	0	0	0	-	-
4 ..	4	4	3	±	0	0	-	-
6 ..	4	4	2	0	0	0	-	-

4 = complete agglutination

* reciprocal of the final dilution of the fluid

TABLE: VII

Effect of Temperature on the Adsorption of Mumps Virus (Enders Strain) with Intact Fowl Cells

Period of adsorption	Tempera-ture	H.A. titre of the supernatant fluid							
		80	160	320	640	1280	2560	5120	10,240
2 hours	0°C	4	1	0	0	0			
	R.T.	4	4	3	0	0			
	37°C	4	4	4	4	4	4	1	0
4 ...	0°C	4	3	±	0	0	0		
	R.T.	4	4	4	2	0	0		
	37°C	4	4	4	4	4	4	2	0
6 ...	0°C	4	4	2	0	0	0		
	R.T.	4	4	4	4	1	0		
	37°C	4	4	4	4	4	4	1	±
0 hour	R.T.	4	4	4	4	4	2	0	0

explanatory notes same as in the table above

TABLE : VIII

The Effect of Variation in the Amount of Fowl Erythrocytes on Adsorption of Mumps Virus (Enders Strain) at 0°C for 2 1/2 hours

Fraction	Percentage of red cells		
	2 %	5 %	10 %
Original fluid	320*	320	320
Supernatant fluid after adsorption	80	80	40
Eluate to original volume	160	240	320

* reciprocal of the H.A. titre (calculated 50% or 2+ haemagglutination end-point)

TABLE : IX

The Use of Different Red Cells as a Means of Concentrating Mumps Virus (Enders Strain)

Original H.A. titre	H.A. titre* after adsorption with					
	2 % fowl cells		2% human cells		2% sheep cells	
	supernate	eluate ø	supernate	eluate ø	supernate	eluate ø
640	120	2560			40	5120
160	40	1280	60	640	10	5120
1280	160	3840			40	10240

* titre expressed as the reciprocal of the final dilution
ø in 1/10th. of the original volume

Table : X

The Effect of Time on Adsorption of Mumps Virus (Enders strain) with Laked Fowl Cells at 0°C

Period of Adsorption (hour)	Haemagglutination titre of the supernatant fluid (reciprocal)							
	40	80	160	320	640	1280	2560	5120
0	4	4	4	4	4	4	44	4
1/2	4	4	4	4	2	0	0	0
1	4	4	4	2	0	0	0	0
2	4	4	4	1	0	0	0	0
4	4	4	4	2	0	0	0	0
6	4	4	4	3	±	0	0	0

Table: XI

The Effect of Temperature on Adsorption of Mumps Virus (Enders strain) with Laked Fowl Cells

Period of Adsorption	Temperature	Haemagglutination titre of supernatant fluid (reciprocal)						
		80	160	320	640	1280	2560	5120
2 hours	0°C	4	4	2	0	0	0	0
	R.T	4	4	4	2	0	0	0
	37°C	4	4	4	4	4	4	0
4 hours	0°C	4	4	2	0	0	0	0
	R.T.	4	4	4	4	2	0	0
	37°C	4	4	4	4	4	3	0
6 hours	0°C	4	4	4	4	0	0	0
	R.T.	4	4	4	4	4	±	0
	37°C	4	4	4	4	4	4	2
0 hour	R.T.	4	4	4	4	4	2	0

TABLE :XII (A & B)

The Use of Varying Strengths of Sheep Cell Suspensions For Adsorption and Elution of the Haemagglutinin and the Complement Fixing 'Viral' Antigen of Mumps Virus (ENDERS strain)

A. Adsorption at 0°C for 1 hour and eluate into half the volume of the original fluid

Fraction	Cell suspension	H.A.test								C.F.test				
		10	20	40	80	160	320	640	1280	5	10	20	40	80
Original fluid	-	4	4	4	4	2	0	0	0	0	0	3	3	4
Supernatant fluid	5%	0	0	0	0	0	0	-	-	2	4	4	4	4
	10%	0	0	0	0	0	0	-	-	2	4	4	4	4
	20%	0	0	0	0	0	0	-	-	2	3	4	4	4
Eluate	5%	4	4	4	4	4	1	0	0	0	1	4	4	4
	10%	4	4	4	4	4	4	2	0	0	1	4	4	4
	20%	4	4	4	4	4	4	1	0	0	0	1	2	4

B. Adsorption at ±1°C for about 16 hours(overnight) and eluate into 1/5th. the original volume

Fraction	Cell suspension	H. A. test							C.Fix.test					
		10	20	40	80	160	320	640	5	10	20	40	80	compl. contr. 1:2,5
Original fluid	-	4	4	4	4	2	0	0	0	0	3	4	4	4
Supernatant fluid	5%	2	1	0	0	0	0	-	2	3	4	4	4	4
	10%	0	0	0	0	0	0	-	2	3	4	4	4	4
	15%	0	0	0	0	0	0	-	2	4	4	4	4	4
	20%	0	0	0	0	0	0	-	3	4	4	4	4	4
	30%	0	0	0	0	0	0	-	4	4	4	4	4	4
Eluate	5%	4	4	4	2	0	0	0	1	2	4	4	4	4
	10%	4	4	4	4	4	1	0	1	3	4	4	4	4
	15%	4	4	4	4	4	1	0	1	3	4	4	4	4
	*20%	4	4	4	4	4	4	2	1	2	4	4	4	4
	†30%	4	4	4	4	4	4	2	0	0	2	4	4	4

* The eluate was reddish due to haemolysis of the red cells --- it was not anti-complementory.

TABLE : XIII

Adsorption and Elution of the Haemagglutinin and the Complement Fixing Antigen of Mumps Virus (James Strain) from Sheep Cells

(Specimen : Dialysed 'Viral' Antigen)

Process and material	Fraction	H. A. test										C . F . test								
		5	10	20	40	80	160	320	640	1280		2.5	5	10	20	40	80	160	320	A.C 2.5
Virus control	at R.T.	4	4	4	4	4	4	4	2	0		0	0	0	2	3	4	4	4	4
	at 37°C (1hr)	4	4	4	4	4	4	4	4	2		0	0	0	1	4	4	4	4	4
	at " 5 hrs.	4	4	4	4	4	4	4	4	2		0	0	0	0	4	4	4	4	4
	at "18 "	4	4	4	4	4	4	4	2	±		0	0	0	3	4	4	4	4	4
Adsorption	Supernat. at 1/4hr.	4	2	1	0	0						0	0	1	2	4	4	4		4
	" 11/2 "	3	2	±	0	0						not tested								
	" 1 "	2	1	0	0	0						0	0	1	2	4	4	4		
	" 1 1/2 "	2	0	0	0	0						0	0	2	4	4	4	4		
	" 2 "	2	0	0	0	0						0	0	2	4	4	4	4		4
Washing of the adsorbed cells	1 st.	0	0	0	0	0						2	4	4	4					4
	2 nd.	0	0	0	0	0						2	4	4	4					4
Elution	at 1/4 hr (6)	4	4	4	4	4	4	0	0	0			1	3	4	4	4			4
	" 1/2 "	4	4	4	4	4	4	0	0	0			0	2	4	4	4			4
	" 1 "				4	4	4	2	0	0			1	3	4	4	4			4
	" 1 1/2 "				4	4	4	2	0	0			1	2	4	4	4			4
	" 2 "				4	4	4	4	1	0			1	1	2	4	4			4
	" 3 1/2 "				4	4	4	4	±	0			1	2	3	4	4			4
	" 7 "				4	4	4	4	0	0			2	2	3	4	4			4
	" 18 "				4	4	4	2	0	0		not tested								

5 % sheep cells used for adsorption
Eluate in a volume equal to the original volume

TABLE : XIV

Adsorption of 'Soluble' Complement Fixing Antigen of Mumps Virus (James strain) by 20 % Sheep Cell Suspension

Fraction	H.A. test				C.Fix. test							
	5	10	20	40	2.5	5	10	20	40	80	160	A.C 1/25
Untreated	4	4	0	0	0	0	0	0	0	tr.	4	4
Adsorbed	0	0	0	0	0	0	0	0	tr	2	4	4
Washing of cells to Orig. volume	0	0	0	0	0	tr	4	4	4	4	4	4
Eluate at 1/2hr.	0	0	0	0	2	3	4	4	4	4	4	4
" " 1 "	0	0	0	0	2	2	4	4	4	4	4	4
" " 2 "	2	0	0	0	tr	1	2	2	3	4	4	4
" " 3 "	4	2	0	0	1	1	>1	2	3	4	4	4

Eluate into original volume

H.A. test : 4 = complete agglutination

C.F. test : 0 = complete fixation
2 = 50 % fixation
4 - no fixation

A.C. = complement control of the antigen

TABLE : XV

Adsorption and Elution of 'Viral' and 'Soluble' Complement Fixing Antigens of Mumps Virus (James Strain) from 5 % Sheep Cells

Specimen	Fraction	H.A.titre (reciprocal)	Titre of C.F. antigen (reciprocal)
Viral antigen (undialysed)	Unadsorbed	160	10
	Adsorbed	40	7.5
	1st.eluate* at p^H7.2	2240	25
	2nd.eluate* at p^H7.8	640	<2.5
Crude C.A.membrane suspension	Unadsorbed	60	30
	Adsorbed	10	20
	1st.eluate▲	130	7.5
	2nd.eluate▲	80	<2.5
Soluble antigen	Unadsorbed	25	20
	Adsorbed	10	20
	1st.eluate*	120	7.5
	2nd.eluate*	80	<2.5

▲ concentrated X 15 times
* .. .. .. X 25 ...

TABLE : XVI

Adsorption and Elution of the Haemagglutinating and the Complement Fixing Antigens of Mumps Virus (Enders Strain) From Sheep Cells

Specimen	Fraction	H.A. testϕ								*C.F. test						
		5	10	20	40	100	200	400	800	2.5	5	10	20	40	80	160
Undialysed pooled allantoic fluid	Unadsorbed	4	4	4	4	4	2	1	0	0	0	0	2	4	4	4
	Adsor./Sc.	4	1	0	0	0	0	0	-	0	0	0	3	4	4	4
	"" /For.	4	4	4	4	4	1	0	-	0	0	0	2	4	4	4
	Eluate/Sc.	4	4	4	4	4	2	0	0	0	0	4	4	4	4	4
Dialysed viral antigen	Unadsorbed	4	4	4	4	4	4	4	2	0	0	0	0	1	4	4
	Adsor./Sc.	4	4	4	4	2	0	0	-	0	0	0	0	0	4	4
	" " /For.	4	4	4	4	4	3	0	-	0	0	0	0	1	4	4
	Eluate/Sc.	4	4	4	4	4	4	2	-	0	1	2	4	4	4	4
3-1/2 day membrane suspem.	Unadsorbed	4	4	4	3	0	0	-	-	0	0	0	0	4	4	4
	Adsor./Sc.	2	0	0	0	0	0	-	-	0	0	0	0	4	4	4
	" " /For.	4	4	4	2	0	0	-	-	0	0	0	0	4	4	4
	Eluate/Sc.	4	4	4	1	0	0	-	-	0	4	4	4	4	4	4
3-1/2 day Soluble antigen	Unadsorbed	4	4	4	1	0	0	-	-	0	0	0	0	4	4	4
	Adsor./Sc.	1	0	0	0	0	0	-	-	0	0	0	0	4	4	4
	" "/For.	4	4	2	0	0	0	-	-	0	0	0	1	4	4	4
	Eluate/Sc.	4	3	1	0	0	0	-	-	2	4	4	4	4	4	4
6-day membrane suspension	Unadsorbed	4	4	4	4	2	0	-	-	0	0	0	0	2	4	4
	Adsor./Sc.	4	4	2	0	0	0	-	-	0	0	0	0	0	4	4
	" "/For.	4	4	4	4	±	0	-	-	0	0	0	0	1	4	4
	Eluate/Sc.	4	4	4	4	±	0	-	-	1	4	4	4	4	4	4
6-day Soluble antigen	Unadsorbed	4	4	4	4	0	0	-	-	0	0	0	0	0	2	4
	Adsor./Sc.	3	1	0	0	0	0	-	-	0	0	0	0	0	4	4
	" "/For.	4	4	4	3	0	0	-	-	0	0	0	0	0	4	4
	Eluate/Sc.	4	4	4	3	0	0	-	-	2	4	4	4	4	4	4

Adsorbed with fresh sheep cells (Adsor./Sc.)
" " formolated " " (Adsor./For.)

Eluates from adsorbed sheep cells; eluted in original volume of buffered (phosphate) saline pH 7.2

ϕ reciprocal of final dilution
* " " " initial "

DISCUSSION

The foregoing experimental results indicate that the mumps virus may be concentrated and purified, by any of the four methods. However, they also emphasize that the choice of a particular method will depend on the specific purposes for which the concentration or the purification is desired.

To obtain a comparatively pure preparation of the virus, free of the components of the egg fluid, the recovery from the urates seem to be the simplest procedure. However, a portion of the virus is lost during the process. Concentration of the virus from embryonic fluids by centrifugation at high speed appears as the ideal procedure for several reasons.

Methyl alcohol precipitation method is effective in concentrating the virus haemagglutinins. It is doubtful whether it is ordinarily possible to free the virus from some of the proteins of the egg fluids. The precipitated virus shows high concentration of the complement fixing antigen. Diagnostic antigens may be prepared in a concentrated form by this method. In fact such antigens prepared according to the methods of Cox et al.(1947) have been used by some investigators in the serological studies on mumps (Aikawa and Meiklejohn, 1949; Florman and Kutch, 1949; etc.).

In/

In view of the findings that the C.F. antigen is not completely recovered in the precipitate although the supernatant fluid does not show its presence, it appears possible that some fraction is altered in an unknown way by the alcohol. If this is true, it may be said by implication that this antigen will not react as effectively as the untreated antigen. Aikawa and Meiklejohn (1949) who used both kind of antigens for comparative study on the serological diagnosis of mumps, have expressed the view that "In general, the allantoic fluid antigen gave higher titres than the viral antigen" (methanol precipitated antigen). It is therefore probable that either there is some denaturation of the precipitated virus, or certain labile factors essential for sensitivity of the C.F. test are removed during the process.

For reasons already explained, the concentrated virus obtained by adsorption and elution from red cells does not appear to be suitable for diagnostic procedures, especially for use as the C.F. antigen. The process however is very effective for obtaining a concentrated preparation of the haemagglutinin. A concentrated eluate prepared by this method was actually used to immunize a fowl and high levels of the antibodies were attained in the convalescent serum.

Amongst the different types of red cells used as a vehicle for adsorption, sheep cells and fowl cells appear to be more effective than the group 'O' human/

human cells. The fowl cells which are highly useful for the concentration of influenza viruses, have been found to be inferior to the sheep cells so far as the mumps virus is concerned. This supports an earlier observation on the effectiveness of sheep cells made by Forster and Carson (1949).

The efficacy of higher percentages (20 to 30%) of the cells for enhanced adsorption has been apparent in all the experiments. In order to purify the soluble complement fixing antigen, of all traces of free haemagglutinin, it appears that the adsorption with sheep cells will be a useful step.

Treatment of infected C.A. membrane suspensions or the soluble antigens with sheep cells does not result in any significant decrease of their complement-fixing titres. Slight reduction in titre, not exceeding a two fold dilution, has been observed in a few cases. This was always associated with the presence of free haemagglutinins (in a low titre) and these were also removed during the adsorption. It therefore seems possible that the soluble antigen may be prepared from crude C.A. membrane suspensions by adsorption with sheep cells, rather than by the high speed centrifugation.

The results of experiments with centrifugation and red cell adsorption and elution are essentially similar to those obtainable with influenza viruses and indicate that the haemagglutinins and the compliment fixing antigens (viral) are the functions of/

of the virus particles. However, it becomes apparent that there is possibly some antigen which fixes complement but, does not cause agglutination. This component therefore remains unadsorbed and only manifests the complement fixing property (table XIII).

In connection with the experiments on high speed centrifugation of the virus in allantoic fluid (table IV) it has been shown that certain fractions with the complement fixing property appear to be sedimented at a slower rate than the haemagglutinins. It may be possible that these fractions are in some way related to the antigen not adsorbing on the red cells. Since the haemagglutinins are thermolabile at 56°C for 10 to 15 minutes, it seems that the presence of some degenerated virus capable of fixing complement but not causing haemagglutination is also responsible for the discrepancy.

Contrary to the conception of the soluble antigen, it was unexpected to find a concentration of the complement fixing antigen in the eluate (table XIV). It is true that the haemagglutinins present in the soluble antigen was in part responsible for the complement fixing property, but that does not explain for the total release of the antigen. Even the washings of the treated cells, showed the complement fixing antigen to a significant level when there was no trace of any haemagglutinin.

It is difficult to explain this phenomenon from the nature of the experiments. But, it seems possible that a part of the soluble antigen is adsorbed on the surface of the red cells in a fashion unlike the attachment of the haemagglutinins. The adsorption is unstable and the antigen appears to come off the cells by ordinary manipulations of washing and resuspension. It is also possible that the antigen is released quickly at a higher temperature and was responsible for the low complement fixing property of the eluates.

SECTION (C)

GROWTH OF THE MUMPS VIRUS IN EMBRYONATED EGGS

As a result of recent investigations on the nature of the mumps virus several factors have been recognised which characterise the virus particles. The virus is endowed with haemagglutinative and infective factors, it fixes complement in presence of homologous antibody and caused haemolysis of erythrocytes from several animal species and man. All these factors develop when the virus is grown in the extra-embryonic fluids of an egg. Besides, it has also been demonstrated that the chorio-allantoic membrane of an infected egg shows the presence of a complement fixing factor ('soluble' antigen) which differs in nature from the mature virus particles of the infected fluid (Henle et al., 1947).

Based on these properties of the virus several laboratory tests have been formulated for the identification of mumps antibody in man and in experimental animals after natural or artificial infections. The presence of the virus or its specific/

specific properties may also be identified by the use of immune mumps sera.

In order to obtain satisfactory antigens for the titration of mumps antibodies and for a number of related studies, it was considered necessary to determine the conditions under which the different properties would attain their maximum function. In this respect, the investigations were extended to a study of the development of above mentioned properties after inoculation of the virus into eggs.

MATERIALS AND METHODS

Virus: The Enders strain was used for all the experiments. The material used as inoculum or seed virus was from different egg passages. The general methods of inoculating the eggs, their management and the harvesting of the embryonic fluids or membranes have been described previously. In some experiments, small amount of the infected fluid was removed repeatedly at intervals from an egg. The inoculation and the subsequent removal of the fluid from these eggs were carried out as follows:

Allantoic fluid:

Method I: The egg was prepared as for the amniotic inoculations, that is, with a window cut in the egg shell on the site of the embryo and covered with 'Scotch tape'. Inoculations were made under direct vision after removing the 'Scotch tape'

Method II: A rectangular portion of the egg shell and the underlying shell membrance (1 cm. x 1·5 cm.) were removed from the air sac end of the egg and the rent was covered with 'Scotch tape'.

A/

A drop of methylated spirit or absolute alcohol placed on the shell membrane overlying the embryo usually shows very clearly the position of the blood vessels and the allantoic cavity. Inoculations were made under direct vision. This method was found to be more satisfactory and quicker than the earlier method and caused minimum injury to the embryos.

In both these methods the inouclations were made with a small-bore record needle (gauge No. 27) fitted to 'tuberculin' syringe. The fluid was withdrawn either with the same kind of needle and syringe or by a glass capillary pipette drawn to fine bore.

Amniotic fluid:

The inoculations were made by the method previously described. The amniotic fluid was removed by using the syringe and needle as described above, after puncturing the amniotic membrane.

The titrations for haemagglutinin, the complement fixing antigen, the infectivity and the haemolysis were carried out according to the method described in Chapter II . Any deviation from the above methods is described in connection with different experiments.

The/

The infected fluids or the egg membranes after removal from eggs were stored at -36°C until the last specimen was obtained. The specimens were usually titrated on the same day in one batch by any particular test. Where this was not possible due to large number of the samples, half of the materials were tested during the middle of the experimental period and the remainder tested at the end, when the last specimen was collected. In each of the experiments the haemagglutination tests were performed with a single batch of fowl cells even on different days.

EXPERIMENTAL AND RESULTS

EXPERIMENT I

Fourteen 7-day old eggs were inoculated with 0·1 ml. of 10^{-2} dilution of the virus* (Enders strain) through the intra-allantoic route. On each succeeding days two eggs were killed and the allantoic and amniotic fluids were removed separately. Altogether 12 eggs were harvested during the 6 days and the rest was discarded. The allantoic or the amniotic fluids collected on each day were pooled and tested for the presence of haemagglutinative property.

The allantoic fluid pools at 24 and 48 hours did not show haemagglutination (H.A.) when tested at 1:10 dilution. At 72 hours the titre of the H.A. test was 1:160. At 96 hours (4 days) the amount of the haemagglutinin showed an increase (H.A. titre = 1:640). The maximum was reached at 5 days (H.A. titre = 1:1280) and the samples at 6 days/

* The inoculum was a pool of infected allantoic fluid with a haemagglutination titre of 1:1280. The virus was in its second allantoic passage.

days showed the same concentration. None of the amniotic fluid pools showed the presence of the haemagglutinin.

The result of this preliminary experiment showed that there is no appreciable growth of the virus, as measured by the H.A. test, during the first two days after the inoculation. The growth is evident on the third day and the same level as the inoculum is reached on the fifth day. It is also apparent that the virus does not grow in the amniotic fluid after the inoculation into the allantoic cavity.

EXPERIMENT II

An experiment similar to that above, was performed with the Enders strain of virus from sixth allantoic passage. Thirty-three 7-day old eggs were inoculated with 0·1 ml. of 10^{-2} dilution of a virus pool (H.A.T. = 1:1280). The eggs were harvested at shorter interval (twice daily) for 6 days and only the allantoic fluids were collected. All the inoculated eggs were living at the time of harvesting.

Each/

Each allantoic fluid was tested for the end point of the haemagglutination titre. The results are shown in table I. The average titre (geometric mean) at different intervals after the inoculation is shown in figure 1.

The results show that the haemagglutinin may be detected within 66 hours after the inoculation with the seed virus. There is a steady increase in the concentration of the haemagglutinin on the following days and the maximum is reached at the end of 5 days. (120 hours). It is also apparent that some of the eggs may show a lower titre when compared with those of the others collected at the same interval. Some of the eggs may not be infected at all. The discrepancy in individual titre is probably less marked towards the end of the incubation period.

The absence of infection in occasional embryos despite inoculation with material causing haemagglutination in over 90 per cent of the remaining embryos has also been observed in connection with other investigations. It is unlikely that such aberrant responses are due to personal error in the technique of inoculating the eggs. Similar results have never been obtained after inoculation of eggs with the/

the Newcastle disease virus or the influenza virus, by employing the same technique of inoculation. Beveridge and Lind (1946) have shown that allantoic fluid from occasional embryos at this age may possess the property of inhibiting the haemagglutination by mumps virus. It is also probable that the embryos were actually infected, but the infectivity of the fluid was of a low titre and did not show the haemagglutination reaction (see the results of experiment VI). Ginsberg et al. (1948a) have observed that the egg fluid inoculated with mumps virus may not contain an appreciable amount of the haemagglutinin although it is still infective at a low titre.

It is important to recognise the presence of aberrant responses during the cultivation of mumps virus in eggs. It shows that unless a large number of eggs are used for any experiment the inoculated egg fluids should always be tested individually for an assessment of the haemagglutinin content.

EXPERIMENT III

The experiment was designed to obtain an evidence of the minimum interval at which the haemagglutinin may be detected. An attempt was made to determine the H.A. titre of the same egg fluid at short intervals after the inoculation.

The inoculating virus was the same as in experiment II, except that it was diluted at 10^{-1} (instead of 10^{-2} as in the previous experiment). Considering the repeated trauma which might be inflicted on the embryo 8-day old eggs were used. The inoculations were made by the method (method I for allantoic inoculation) described earlier in this section. Four eggs were inoculated intra-allantoically. Intra-amniotic inoculation was also made into two eggs. The egg fluids were titrated immediately after they were obtained, using a minimum dilution (final) of 1:10.

The results are shown in table II. It will be seen that no haemagglutinin is detected in the allantoic fluid 12 hours after the inoculation. Within 36 hours there is definite evidence of haemagglutination in all the four eggs inoculated through the allantoic route. While two of the egg fluids (No. I and IV) show higher H.A. titre at the/

the end of 48 hours, one (No. II) does not show further rise. The H.A. titre of the latter does not attain a high level even at the end of 84 hours. As compared with the results of experiment II, it appears that the haemagglutinin develops earlier and also reaches a high titre in shorter time if a concentrated dose of the virus is used as an inoculum. The final yield of the virus at the end of 5 days is probably not significantly higher than that in the former experiment.

The amniotic inoculations showed the presence of the haemagglutinin in one of the eggs at the end of 36 hours. By 48 hours both eggs showed a sharp rise in the content of the haemagglutinin. There was at least 32-fold increase in the titre of the fluid from egg No. I and 64-fold increase in the other fluid. It was striking that a titre comparable to that of the inoculum was reached within 48 hours after the inoculation.

EXPERIMENT IV.

This experiment was performed to observe the effect of injecting a diluted inoculum of the virus. A pool allantoic fluid injected with the Enders strain of virus and with a low H.A. titre of 1:320 was selected. It was infective to eggs at 10^{-5} but not at 10^{-7}. A 10^{-3} dilution was used as the inoculum.

Five 8-day old eggs were inoculated intra-allantoically by the method No.II, using 0·1 ml. as the inoculating dose. The allantoic fluid was removed daily from each of the eggs, starting at 48 hours, and examined for the presence of the haemagglutinin. The 48 hours and 72 hours samples were examined at 1:10 and 1:20 dilutions only, immediately after they were collected. The samples withdrawn at 96 and 120 hours were preserved at 4^{0}C after an initial dilution of 1:10 in normal saline and tested along with those collected at 144 hours.

The results of the titrations are shown in table III. No haemagglutinin was detected in any of the egg fluids at 48 hours (titre less than 1:10). At 72 hours only 3 out of 5 fluids showed positive H.A. at 1:10, including one at 1:20. All the fluids showed a rise of H.A. titre at 96 hours. After the maximum was reached at 120 hours there was a tendency of/

of the titres to decline at 144 hours. The growth-curve of the haemagglutinin is shown in fig. I.

It is apparent from this experiment that as a result of injection of a diluted inoculum the earliest evidence of the haemagglutinin in allantoic fluid is comparatively delayed. There is however no appreciable difference in the yield of the titres at the end of 5 days. In fact, the titre of the newly formed haemagglutinin may exceed that of the inoculum. The tendency of the decline in titre at 6 days is probably due to the presence of viscid albuminous material, which has often been observed in the allantoic fluid of the embryos at this age. The discrepancy in the titre of individual egg fluids is also apparent.

EXPERIMENT V.

The experiment was undertaken to determine the nature of the development of complement fixing principles ("viral" and "membrane" antigens) after intra-allantoic inoculation of the mumps virus. The findings were also correlated with the appearance and development of the haemagglutinin.

The Enders strain of virus, from the 42nd. allantoic passage (diluted 10^{-2}) was used as inoculum. The virus pool had a H.A. titre of 1:240. Seventeen 8-day/

8-day old eggs were inoculated, each receiving 0·1 ml. of the virus dilution. Two eggs were harvested at a time at different intervals till 96 hours. Afterwards only one egg at a time was harvested at 108, 120 and 132 hours.

The allantoic fluid and the C.A. membrane from each egg were collected separately. The membrane was thoroughly washed in large quantity of buffered-saline to get rid of the urate deposits and any free virus particles and albuminous materials, as far as possible. The fluid and the membrane were preserved at -36°C till all the samples were collected.

Each of the allantoic fluids was tested separately, and on the same day, for presence of the haemagglutinin. A 20% suspension of each of the C.A. membrane was prepared in sodium azide solution (normal saline containing 0·08% sodium azide) and clarified by spinning in an angled centrifuge. The method followed was the same as that used for the preparation of 'soluble' antigen for complement-fixation tests, except that the centrifugation was carried out at 4,000 r.p.m. for 10 mins. Only the cellular debris was thrown down. The supernatant suspension consisted of the total virus materials (the haemagglutinin and the complement-fixing antigen/

antigen) liberated from the crushed membranes, including the true 'soluble' antigen as described by Henle et al.(1947).

The complement-fixing antigen content of the allantoic fluids and the membrane suspensions was determined by titration against known mumps anti-sera. A pool of convalescent sera from mumps patients diluted at 1:4 was used for this purpose. All the specimens were tested on the same day. Equal volumes of the allantoic fluids or the suspension of C.A. membranes harvested at different intervals were pooled before the test. Necessary controls were also put up, including the complement control and the control with normal human serum, containing no mumps antibody. The test was completed according to the method described in chapter II .

The haemagglutinin content of the allantoic fluids is shown in table IV. Its rise is represented graphically in fig. 2. As in two earlier experiments, the haemagglutinin is detected on the 3rd. day (72 hours) after the inoculation and rises to a maximum on the 5th day. The discrepancy in the H.A. titre of individual egg-fluids, as previously noted, is also observed in this experiment. Thus, during the tests, some of the tubes showed irregular and atypical haemagglutination e.g. in eggs 7 and 8.

The/

The irregular reaction when present is observed only in some of the samples of allantoic fluid collected at a time preceding the appearance of fully formed haemagglutinins.

Each of the C.A. membrane suspension was tested for haemagglutinin content. The result of the H.A. titration is shown in table IVa.

It will be observed that at 12 hours the membrane suspensions showed the presence of haemagglutinins in small amounts. Although the agglutination of the red cells was not typical and complete, there is no doubt that they were evidence of positive reactions. The result was unexpected because in none of the other experiments in this series the haemagglutinating or infective virus could be detected in the allantoic fluid at this hour.

Earlier observations on the nature of development of the bacterial viruses (Delbrück, 1947) and the influenza virus (Hoyle, 1948; 1950; Henle and Henle, 1949b) suggest that the virus particles when they infect a cell are altered by some unknown processes and cannot be identified by the available methods.

Thus/

Thus, it is improbable that the present finding was a result of the multiplication of the virus inside the chorio-allantoic cells. Moreover, the haemagglutinin could not be detected in other membrane suspensions even up to 84 hours. The available evidence therefore implicates that the virus was adsorbed on the surface of the cells, before gaining access inside them, and were released during the preparation of the membrane-suspension. The incomplete haemagglutination was due to the presence of inhibitors in the tissue extract.

The haemagglutinin content of the membrane gradually increased from 96 hours and reached the maximum at 132 hours ($5\frac{1}{2}$ days) when the experiment was terminated. It was also observed during the titrations that occasional tubes at higher dilutions showed evidence of partial haemagglutination ($\pm$, 1^+ or 2^+reactions). The reactions were similar to those observed in some of the infected allantoic fluids.

Development /

Development of the C.F. antigen and correlation with that of the haemagglutinin:

The results of C.F. tests with the allantoic fluids (viral antigen) and the membrane suspensions (membrane antigen) are shown in tables Va and Vb.

It will be seen that during the initial 60 hours after inoculation, there is no increase of the viral and the membrane antigens. The haemagglu-:tinins are also absent from the fluid and the membrane. It appears that during this period (latent period) the virus probably grows inside the cells of the membrane, but cannot be measured by the H.I. and C.F. tests.

The growth of the virus is more marked during 60 to 72 hours. Some of the virus is liberated into the allantoic fluid and this is characterised by the sharp increase in the haemagglutinin content. It is also apparent that there is an increase of the complement fixing antigen inside the membrane, although there is no parallel rise of the same in the allantoic fluid. This anomaly of the differential rise probably indicates that the intitial virus, that is, the newly formed virus which is liberated in the allantoic fluid can only cause haemagglutination of the red cells and lack the complement fixing property.

The /

The complement fixing property of the virus is manifested in the allantoic fluid at least 12 hours after an increase of the same inside the C.A. membrane. Later, there is further concentration of the virus inside the membrane and more is liberated into the allantoic fluid. The fluid shows an increase of both the haemagglutinin and the complement fixing antigen.

It is noteworthy that at least in two samples collected at 72 and 84 hours the membrane suspension showed a significant increase in the C.F. antigen, whereas the haemagglutinins could not be demonstrated. This apparently shows that the latter are formed in the membrane at least 12 to 24 hours after the development of the C.F. antigen. It is however doubtful whether this time interval is real. Some of the membrane suspensions, especially those at 84 hours show partial haemagglutination in the lower dilution tubes. It is possible that haemagglutinins present in low amounts in the suspension were partially masked by the inhibitors present in the extracts of the membrane.

The sequence of events between 108 and 130 hours cannot be examined critically, because only single eggs were examined during the interval. However/

However, it may be pointed out that the relative contents (or the ratio) of the haemagglutinin and the complement fixing antigen in the membrane or the allantoic fluid are comparable.

It will be observed in the graphs of figure 2 that at 108 hours there is an increase of the virus in both the allantoic membrane and the fluid. At 120 hours it appears as if there is a large shower of the virus particles from the cells of the membrane. The antigen content of the membrane falls sharply and there is a simultaneous increase of the C.F. viral antigen in the allantoic fluid. Further increase of the haemagglutinin at this period also supports the above view. The slight increase of the haemagglutinin in membrane suspension may appear paradoxical; but it is quite likely that some of the discharged virus which manifests more of the haemagglutinating property has adsorbed on the cells of the C.A. membrane (C.F. 12 hours samples) and is responsible for the increase.

The reasonability of the above view is supported by the findings at 130 hours. There is a decline of the haemagglutinin and the C.F. antigen content of the allantoic fluid after the sharp rise at 120 hours. The loss of the virus particles from allantoic fluid is readily explained by assuming that/

that they have been adsorbed on fresh uninfected cells of the C.A. membrane. In fact, the membrane suspension at 130 hours shows a sharp rise in the titres of both the H.A. and the C.F. tests.

EXPERIMENT VI

The experiment was undertaken to determine the growth of the mumps virus with reference to its following properties:

(1) Haemagglutination

(2) Complement fixation

(3) Infectivity

(4) Haemolysis

Fifty 8-day old eggs were inoculated with the Enders strain of the virus. The inoculum was 0·1 ml. of 10^{-2} dilution of a pool of allantoic fluid from eighteenth passage. Two eggs were harvested at 12 hours for the allantoic fluid. Thereafter, every 12 hours 3 eggs were harvested for the first six days after the inoculation. On 7th, 8th and 9th day 3 eggs were harvested daily. The remaining eggs were discarded.

The allantoic fluid from each egg was collected separately in screwcapped glass vials and stored at -36°C till the last sample on the 9th day was collected. The C.A. membranes were also harvested from each egg. They were washed in sterile buffered saline as in experiment V and preserved at -36°C in the same way as the corresponding allantoic fluids.

The allantoic fluids, together with the inoculum were examined for the content of the mumps virus in term of different properties as mentioned above.

The/

The tests used for this purpose and their results are described separately as below.

(A) Haemagglutination test:

There were 44 specimens of the allantoic fluid. Each fluid was tested individually at dilutions (final) from 1:20 to 1:2560. All the fluids were examined on the same day with 0·5% fowl cell suspension at room temperature.

The results of these titrations are shown in table VI. The geometric mean titre of the fluids removed at intervals is shown in figure 3(d). The results are correlated with the time after inoculation and compared with those obtained by other tests. In the figure, the heavy black line passes through the titres of the pooled fluids harvested at 24 hour intervals. The dotted line shows the variation in the titre when all the samples obtained at 12 hour intervals are considered.

It will be found that after the inoculation of the 'seed' virus (H.A.T. = 1:640) there is no detectable haemagglutination at 12 hours. Up to 60 hours no haemagglutinin is present in the allantoic fluid. It is detected at 72 hours and there is a sharp rise between 60 to 84 hours. Afterwards there is a steady increase of the haemagglutinin with/

with slight variation and the level is maintained till the end of the experimental period.

Certain peculiarities of the haemagglutinin referred to in the earlier experiments were noted again.

(a) In many of the allantoic fluids harvested between 12 and 60 hours, partial haemagglutination was observed in some of the higher dilution tubes (see table VI). They were again evident on repeating the tests (see experiment V, table IV, egg No. 7 and 8). These partial H.A. reactions at 60 hours might be due to the presence of newly formed haemagglutinin; it is possible that if tested at lower dilutions these fluids would show the haemagglutinins. Similar reactions were however absent when 2% fowl cells were used for the titrations and the test was carried out at 4°C (as for 'haemolysis test', see below).

(b) Two out of three allantoic fluids removed at 96 hours did not contain any haemagglutinin, although the other fluids removed between 72 to 216 hours were positive. A similar observation was made in experiment II, in which one of the allantoic fluid removed at 96 hours was also negative. A pool of the two negative fluids, in this experiment, was however found to infect eggs at a dilution of 10^{-3}. The significance of this reaction will be discussed later.

(c)/

(c) After 120 hours (5 days), there was a slight tendency of the H.A. titre to decline (figure 3). A similar fall in titre was noticed at this time in experiments No. IV (see figure 1) and No. V (see figure 2).

It should be mentioned in this connection that there is a progressive reduction in the volume of the allantoic fluid from 7 days onwards, that is, when the embryos are older than 14 or 15 days. Therefore, the maintenance of the H.A. titre at a high level is more a relative than a true indication of the concentration.

(B) Complement fixation test:

The allantoic fluids collected at 24 hour intervals, after the inoculation, were mixed in equal volumes to prepare eight different pools. Another pool was made from two samples collected at 12 hours. These 9 pools together with the 96 hour sample which showed a positive H.A. reaction and the inoculum were titrated for their content of the complement fixing antigen.

The titrations were made against the mumps antiserum from a rabbit, immunised by repeated inoculations of the Enders strain of the virus. Necessary controls were also included especially the complement control/

control and the control against normal rabbits serum for each pooled fluid. Both the normal serum and the immune serum were used at a dilution of 1:8. They were found satisfactory by previous titrations and did not show any anti-complementary property or non-specific fixation with normal allantoic fluids (from 12 to 16 days old embryos). All the pooled fluids were tested on the same day.

The results of the titrations with the anti-serum is shown in table VII. As the controls were satisfactory, their results have not been included in the table. The titre of each of these samples have been converted into the negative log values (log unit) and shown in the graph of figure 3.

It will be found that there is no detectable antigen in the allantoic fluid between 12 and 48 hours after the inoculation. It is just perceptible at 72 hours and increases rapidly for another 24 hours. The rise is gradual for the next 48 hours and a maximum level is reached at the end of 144 hours (6 days). There is a slight tendency of the titre to decrease in the following two days with a negligible rise at the end of 9 days.

The results are in general agreement with the previous findings, in experiment V (see figure 2), except/

except that the tendency of the decline in titre was observed at 24 hours after, in this experiment. A peculiarity noticed during the titrations was that in many of the pooled fluids there was 'zoning' and the end points were not usually clear cut. The zoning was marked in the 216 hour sample, obtained from 17 day old embryos.

(C) Infectivity titrations:

The allantoic fluids harvested at intervals were mixed in equal volumes to prepare pooled samples. Along with the inoculum these pools were titrated for the infectivity titre (E.I.D.50). Out of the 3 allantoic fluids collected at 96 hours, only one which showed positive H.A. titre was tested for the infective capacity.

8-day old eggs were used for all the titrations. The inoculated eggs were incubated for 5 days. The allantoic fluids were tested for the growth of virus by H A. test at 1:10, 1:20 and 1:40 dilutions. An inoculated egg was regarded as infected if any of the above dilutions showed more than 2+ reaction. The other techniques of performing the tests were the same as described in chapter II.

For the pooled allantoic fluids collected at 12 hours to 60 hours, the dilutions were made from 10^{-1}/

10^{-1} to 10^{-6}. For the rest of the samples the range of the dilutions were from 10^{-2} to 10^{-7}. A minimum of 4 eggs were inoculated for each dilution, the inoculating dose being 0·1 ml. The E.I.D.50 has been expressed as the dose per ml. of the fluid.

The results of the titrations are shown in table VIII. The titres have been presented in the graph of figure 3. In this figure the end points of the 24-hourly samples are joined by a bold line. The dotted line indicates the level when 12 hourly samples are considered.

It will be found that within 12 hours after the inoculation the infective virus has disappeared from the allantoic fluid. The pool at 36 hours is also negative. A large amount of the infective particles is liberated into the allantoic cavity between 36 and 48 hours, and a maximum infective capacity is reached within 96 hours. It is apparent from this period of the growth, that, at least 24 hours before the H.A. test becomes positive, infective virus particles may be demonstrated in the allantoic fluid. The infectious capacity also reaches its maximum before the H.A. titre reaches its height.

After 96 hours there was a drop in the infectivity titre/

titre for about 36 hours. The titre again reached a high level 48 hours after the initial peak (144 hour sample), to be followed by another fall in the titre. The variation in the titre is probably of some significance.

The sudden fall in the infective titre (below 10^{-2} dilution) at 108 hours, even when the presence of the virus could be demonstrated by other test, was further investigated. The titration was twice repeated with the same negative result. The C.A. membranes collected at 108 hours were pooled and made into a 20% suspension with sterile buffered saline. The suspension, lightly clarified by spinning at low speed, was injected into 15 eggs at 10^{-1}, 10^{-2} and 10^{-3} dilutions. All the eggs were found to be infected after 5 days' incubation.

This experiment showed that a large amount of infective virus was still present in the C.A. membranes although the corresponding allantoic fluids were non-infective. Unfortunately, comparative titrations of the virus in other C.A. membranes could not be made, so that it is not possible to assess the significance of the infective capacity of the 108 hour membrane suspension.

The variation of the infective titre after 96 hours may be explained as follows. It has been shown/

shown earlier that for 36 hours after the inoculation of the seed virus there is intracellular growth of the virus so that no infective particles are present in the allantoic fluid. The first batch of the infective virus is liberated within 48 hours. It appears, therefore, that to complete a cyle of the growth, it takes about 36 to 48 hours.

At 96 hours after the inoculation the embryos were 12 days old. It is known that from about this time onwards there is a rapid development of the embryo along with its covering membranes. Therefore it is reasonable to believe that there is also an increase in the number of the cells lining the allantoic cavity. The newly formed or the remaining uninfected cells are soon invaded by the freshly discharged virus particles, so that there is a considerable reduction of the infective particles. The second cycle of the virus growth now takes place and within 48 hours after the first 'peak' at 96 hours, that is, at 144 hours after the inoculation there is again a high rise in the form of a second peak.

The sharp fall in titre after 96 hours is comparable to what happens after the inoculation of the seed virus. Since some of the cells in the membrane are/

are already loaded with the virus, it is expected that the second peak would be reached within a shorter time due to simultaneous release of the virus from the younger and the older cells.

The same sequence probably follows the second peak at 144 hours. In addition, with the increasing age of the embryo other extraneous factors also cause increasing inactivation of the virus particle. As a result, the 3rd peak seen at 216 hours is not so high as the earlier ones. The fall in the infective titre after 144 hours also correspond with the declining titres of the virus content as measured by other tests.

(D) Haemolytic test:

The allantoic fluids were mixed to form different pools as described for the infectivity test. The pooled fluids and the inoculum were titrated for the haemolytic activity by the method described in chapter II . A pool of allantoic fluid from 14-day old normal (uninoculated) eggs was also included as control.

The results of the haemagglutination test with 2% fowl cells and the haemolytic activity of the different pools, inoculum and the control are shown in table IX. The haemolytic activity is expressed in/

in terms of the colorimeter reading - as a difference between the direct reading and the mean reading of the saline control tubes.

The significance of the variation in colorimeter readings has been discussed in chapter III, in connection with the standardisation of the test. The following facts should be emphasised in this connection. (1) In serial 'blank' titrations with normal saline, the maximum difference in the colorimeter readings was 0·030. This indicates that a difference between any two consecutive readings in a serial titration beyond ±0·15 may be taken as significant. (2) During the 'blank' titrations the readings in every case was above 'zero', that is, there was evidence of a trace of haemolysis in every tube. The haemolysis was expected, because the cold incubation at 4°C, prolonged incubation at 37°C, shaking, centri fugation etc., all lead to the destruction of a few red cells. For this reason, any negative value of the colorimeter reading (where the mean reading of the saline control is higher than the value obtained in the test proper) may be taken to indicate that there has been some inhibition of the haemolysis.

The/

The amount of virus present in a fixed dilution (1:10) of the different allantoic fluid pools, as determined colorimetrically in term of the haemolytic activity, is shown in a graph (figure 3a). The colorimeter readings have been obtained from table IX. The graph correlates the haemolytic activity, that is, the haemolysin content and the period elapsed after the inoculation of the eggs with seed virus.

It will be observed that 12 hours after the inoculation there is some amount of haemolytic activity of the allantoic fluid. This probably is not related to the virus activity, because the normal allantoic fluid may also show some haemolysis at this range (table IX).

Between 12 and 60 hours there is no significant amount of the haemolysin, which indicates the absence of free virus in the allantoic fluid. The nature of the curve also indicates that the allantoic fluid at this age of the life of the embryo has some inhibitory effect on the haemolysis of the red cells. The results of the infectivity titrations have shown that free infective virus is present in the allantoic fluid at least in the 48 hours and 60 hours samples.

It/

It is apparent therefore that, either (i) the allantoic fluid masks the presence of the virus due to the inhibitory factors present in it, or (ii) the newly formed virus is only endowed with the infective property and lacks the factor or factors which is responsible for causing haemolysis.

At 72 and 84 hours, simultaneously with the increase of the haemagglutinin and the complement fixing property, the haemolysin is also detected at significant level. It is probable that at this interval after the inoculation, the virus particles are more mature so that they exhibit the haemolytic activity. If the inhibitory factor in the allantoic fluid is still operative at this stage, it is possible that they are inactivated by the mature virus.

The decline in the titre of the haemolysin at 96 hours appeared somewhat paradoxical, since the allantoic fluid showed the presence of virus by all the other three tests. It will be seen in table IX and figure 4 that when the allantoic fluid is diluted to 1:20, instead of 1:10, the colorimeter reading indicates the presence of virus. This suggests that the inhibitory factor present in the allantoic fluid is effective at least up to a dilution of 1:10 at/

at this age of the embryo (12 days). In fact, the inhibitory titre is much higher and is more apparent when the virus content is considerably decreased by diluting out the allantoic fluid. Further evidence on the presence of the inhibitory factor will be discussed later.

After 108 hours there is a sharp rise of the haemolysin content and a high level is reached at 132 hours (between 5 to 6 days). This is followed by another cycle of a fall and then a rise of the haemolysin content. The pattern of the curve between 132 and 216 hours resembles closely that of the infectivity and in some respect that of the complement fixing property.

It is probable that the factors mentioned in connection with the infectivity titration are also responsible for the variation in the content of the haemolysin. Moreover, the presence of considerable amounts of urates and celluler debris, alteration in the p^H of the allantoic fluid, and presence of inactive virus (Chu and Morgan, 1950 a) may also be responsible for the fall of the haemolysin titre.

It has been pointed out earlier, that, under the experimental conditions some amount of haemolysis of/

of the red cells may take place, even when they are suspended in buffered normal saline (natural haemolysis). During the titrations it became apparent that the allantoic fluids from eggs, $8\frac{1}{2}$ to 12 day old, usually exerted some inhibitory effect on this natural haemolysis. The haemolysis could be observed even up to a high dilution of the allantoic fluid, although, it must be said that the results were sometimes irregular (table IX). With the increasing growth of the virus the inhibitory effect was less marked. As mentioned earlier, it was however apparent when the amount of the virus decreased due to dilution of the fluid.

The nature of this inhibitor has not been investigated any further. Its presence has been recognised by Chu and Morgan (1950,b). It is probable that among other factors the inhibitors of haemagglutination present in normal allantoic fluid (Anderson, 1949) are also effective against the haemolysin.

It is obvious from the above results that the haemolytic activity of the different allantoic fluids is closely associated with the amount of the virus present in each. It is in fact a function of the virus/

virus particle itself. In spite of certain similarity in the nature of the development of the haemolysin and other properties, there are indications that the haemolytic activity is independent.

The haemolytic test appears to be more sensitive than the H.A. and C.F. tests although less so than the infectivity titrations. It is however unduly influenced by the presence of certain factors present in the allantoic fluid of developing embryo.

EXPERIMENT VII

The variation in the yield of haemagglutinin in different eggs, after an inoculation of the same dose of virus, was examined with 8-day old embryos. A 10^{-3} dilution of the virus (42nd allantoic passage, Enders strain) was inoculated into the allantoic cavity of 16 eggs, each of which received a dose of 0·1 ml. The eggs were incubated for $5\frac{1}{2}$ days at 35°C after which the allantoic fluids were tested at 1:20 dilution for the presence of haemagglutinin.

One/

One of the egg fluids was found negative. The remaining fluids were titrated individually for the end point, by H.A. test.

The results of this experiment are given in table X. It will be observed that a majority of the fluids show a particular titre (1:400). However, there are some which may show a lower or a higher titre. A four-fold difference could be found between the maximum and the minimum titres.

It has been shown in chapter III that if a sample of infected fluid is titrated several times on the same day, in about 25% of the cases a difference of only two fold in the titre was observed. In no case was a four fold difference observed. It is therefore apparent from this experiment that a significant variation in the yield of the haemagglutinin may result even after the inoculation of the same amount of virus in different eggs.

EXPERIMENT VIII

The influence of the dilution of virus containing allantoic fluid used as inoculum was examined with 8-day old eggs. The dilutions studied were 10^{0}, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. Two such experiments were performed with the same virus pool (42nd passage). 0·1 ml. of different dilutions of the virus was inoculated into the allantoic cavity of two big batches of eggs. In experiment "A", the eggs were harvested after 5 days of incubation and in experiment "B" they were harvested after 6 days. The positive fluids from each batch of eggs inoculated with the same dilution of the virus, were pooled in equal amounts and tested for the end point of the H.A. titre.

The data are presented in table XI. It will be observed that there was a tendency for a better average yield of the virus when the seed inoculum was diluted, e.g. at 10^{-2} and 10^{-3}. However, with further dilution (at 10^{-4}) there was less production of the haemagglutinin. The experiment also shows that the titres at 6 days are, in general, lower than those at 5 days. A similar observation has been made in earlier experiments.

Besides/

Besides the examination of the pooled materials in the experiment "A", each positive fluid was also tested for the individual end point. The calculated 50% haemagglutination titre (2+) of each fluid and the geometric means are shown in table XII.

As in the experiment VIII, it will be observed that there is a variation of the end point of the H.A. titres. High individual titres are evidently obtained at 10^{-2} and 10^{-3} dilutions. The geometric mean however does not reflect a significant difference of more than 2-fold in titre, between the minimum and the maximum values.

It could be said, therefore, that various dilutions of the seed inocula may result in a difference in the yield of the haemagglutinin, but, when large number of fluids are pooled a marked difference may not be apparent. It is however obvious that with the use of diluted inocula, such as 10^{-2} or 10^{-3}, there is a greater chance of obtaining a pool with higher haemagglutinin content.

DISCUSSION/

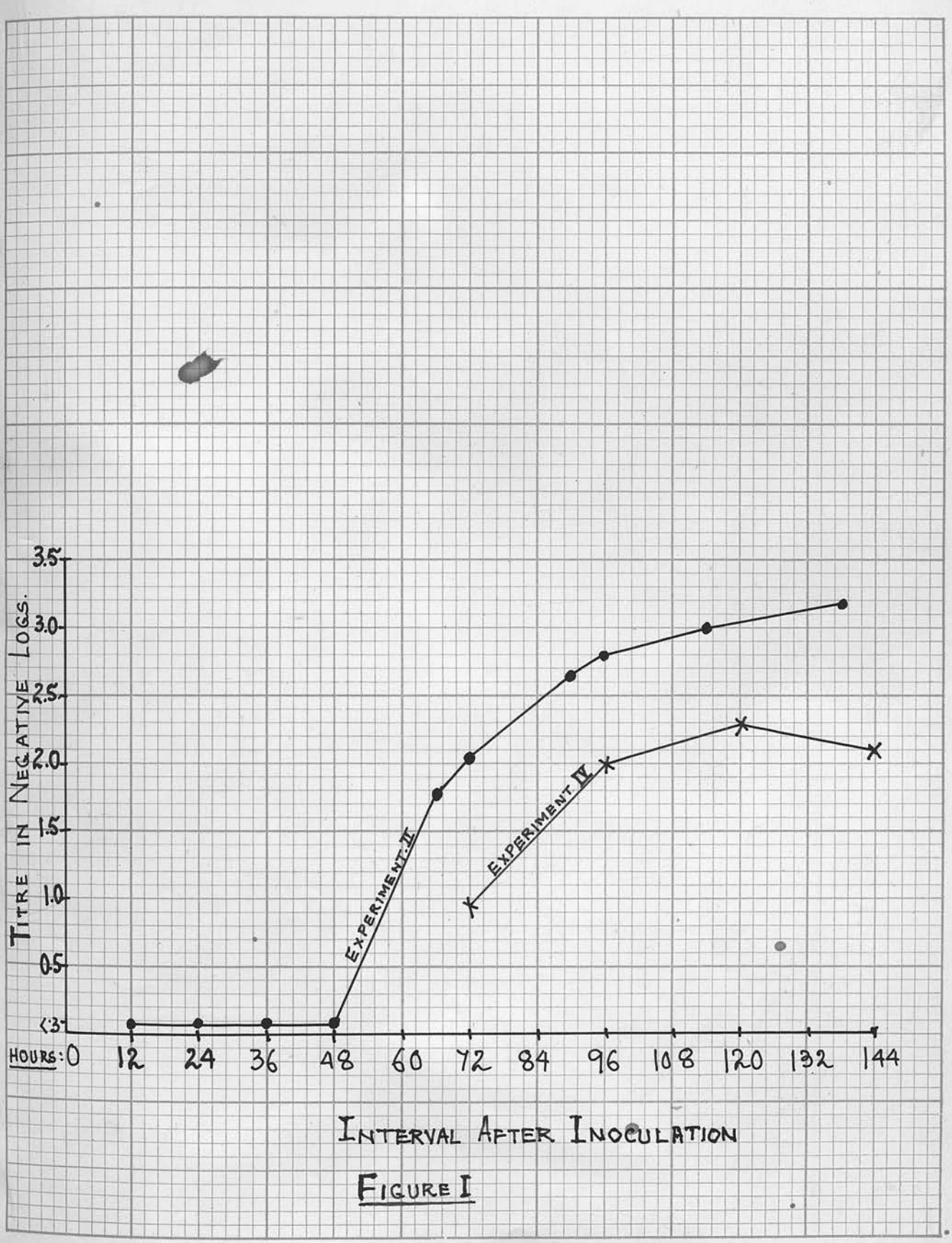


FIGURE I

Development of the Mumps Haemagglutinins

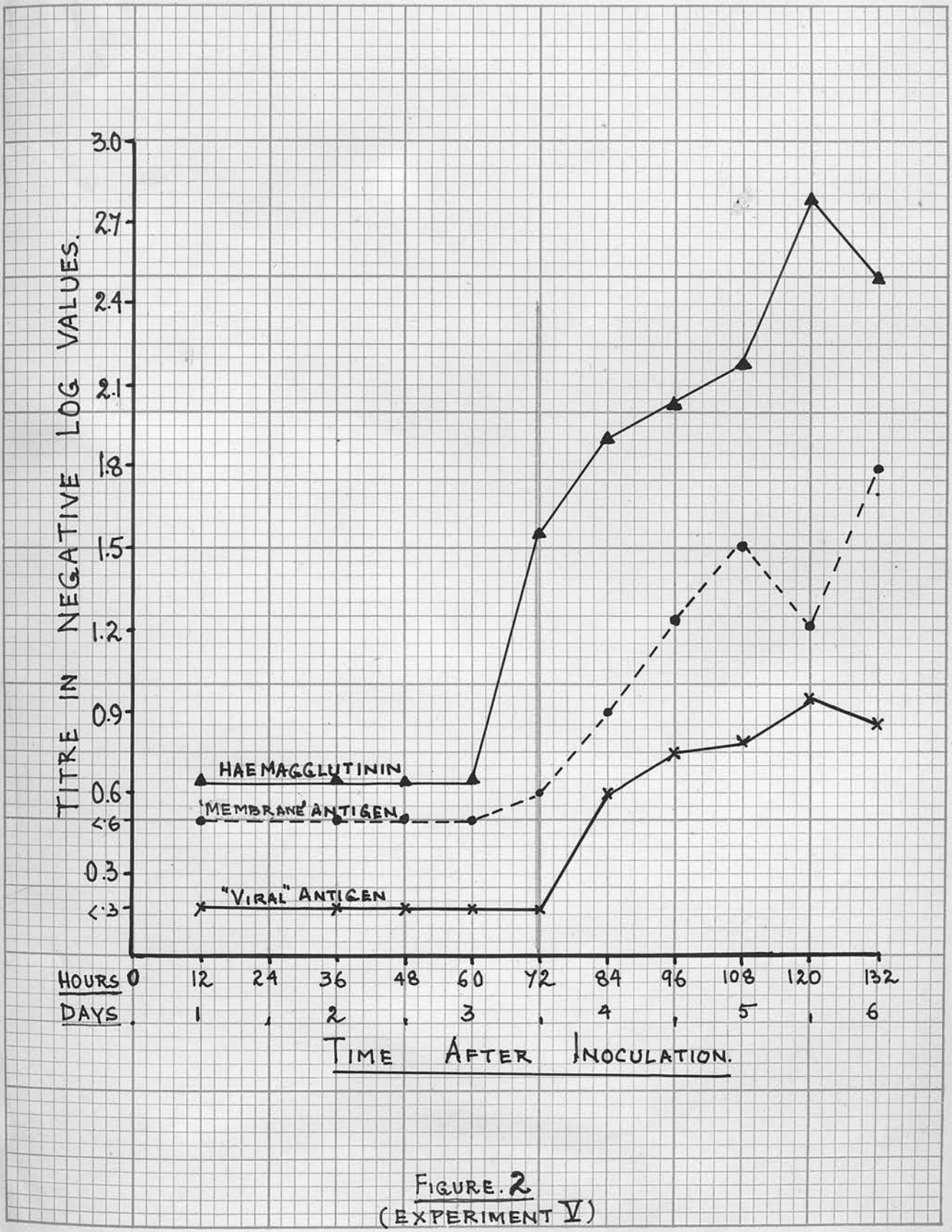


FIGURE. 2
(EXPERIMENT V)

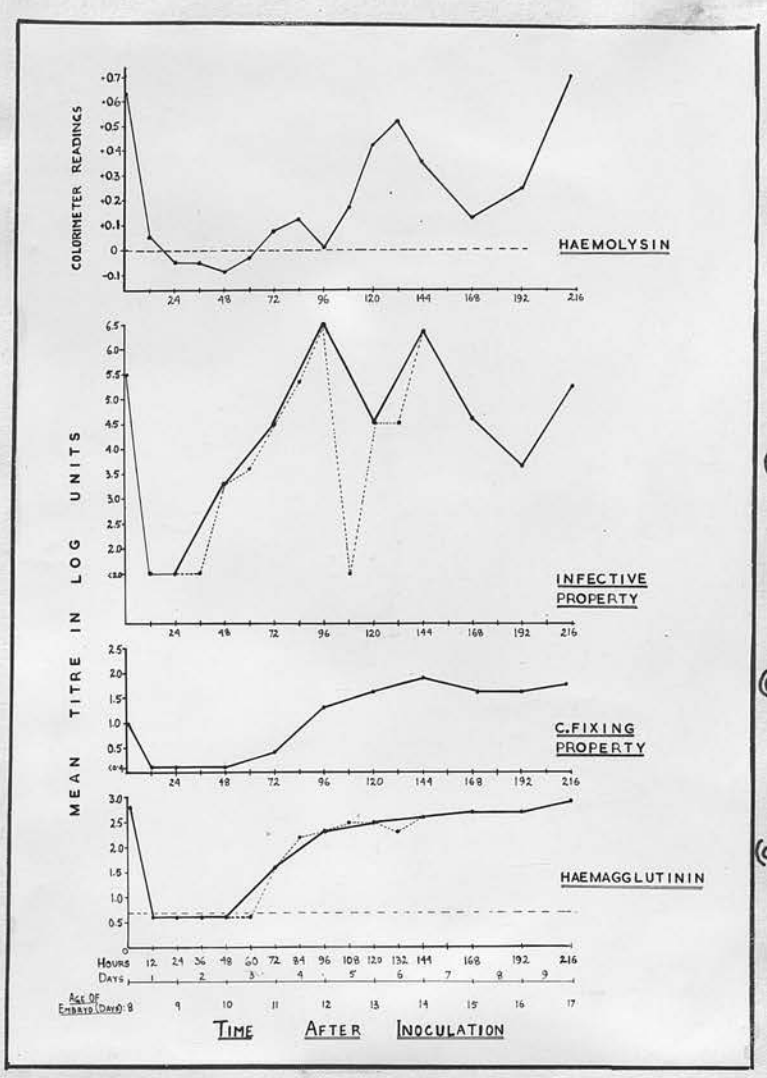


Figure 3.

...Graphs showing the appearance and subsequent variations of different properties of the mumps virus (Enders strain) after inoculation into 8-day old embryonated eggs.
...Values for the inoculum are shown at 0-hour.
...In (b) and (d) the dotted lines pass through all the results of samples collected at intervals; the heavy black lines pass through values at 24-hour intervals for that period.
...The horizontal broken line in (d) shows the lowest titre at which different samples were examined.
..In (a) it passes through the "zero" value of the colorimeter.
...For other explanations see text.

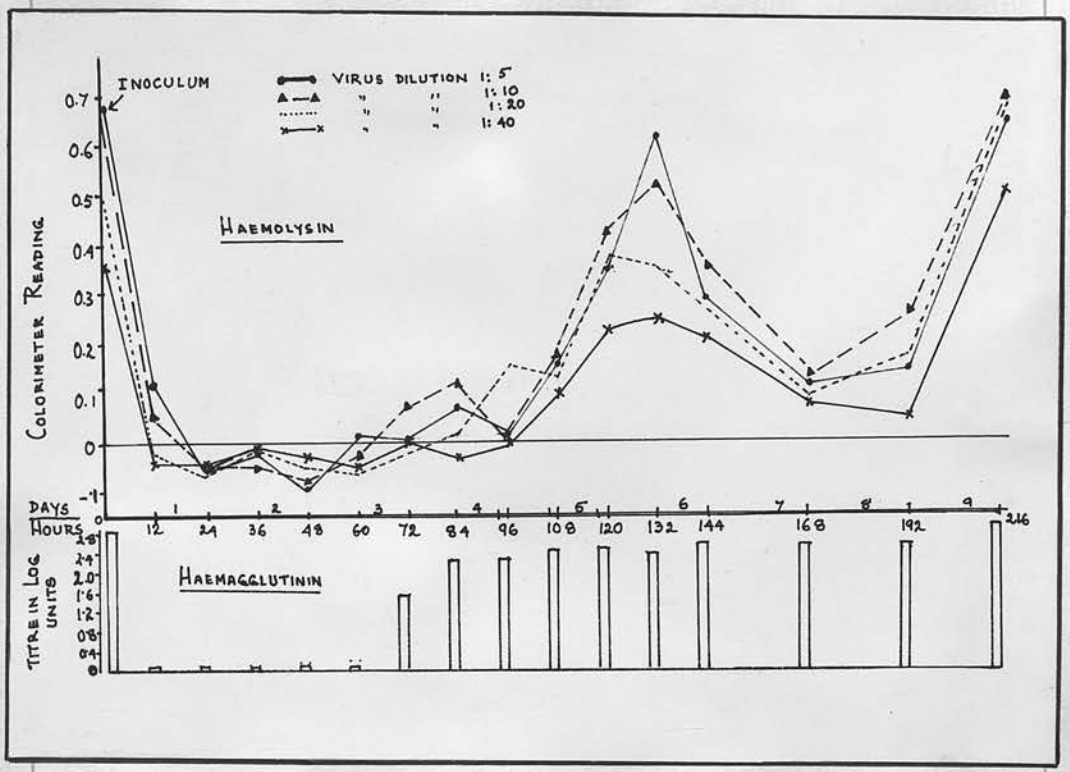


Fig. 4 .

Correlation of the time of appearance and the variation of haemolytic and haemagglutinating properties of the mumps virus (Enders strain) after intra-allantoic inoculation in 8-day old embryonated eggs.

The nature of the graphs between 60 and 96 hours samples gives an indication that some factor or factors present in the allantoic fluid of 10 to 12-day old eggs have inhibitory effect on the haemolytic property of the mumps virus.

TABLE : I

The Increase of H.A. Titre in Eggs After Inoculation of the Mumps Virus* (ENDERS Strain) into the Allantoic Cavity

(EXPERIMENT : II)

Time after inoculation (hours)	Reciprocal of the titre in 3 allantoic fluids			Geometric mean (log)
18	0	0	0	0
24	0	0	0	0
42	0	0	0	0
48	0	0	0	0
66	0	160	20	-1.75
72	320	20	400	-2.03
90	200	800	500	-2.63
96	400	0	1000	-2.8
114	1000	1000	1000	-3.0
120	1000	1500	1500	-3.12
138	1500	1500	1500	-3.17

* Inoculum : diluted 10^{-2} (H.A. titre = 1:1280)

N.B. 1) Where the H.A. titre was less than 1:10, the minimum dilution used for the test, the value of the reciprocal has been taken as zero.

2) The mean(geometric) value has been calculated only from the results of the positive tests

— — — —

TABLE : II

Developement of the Haemagglutinin in Individual Eggs After the Inoculation With Mumps Virus ϕ

(EXPERIMENT : III)

Route of inoculation	Egg NO.	Days after inoculation							Remarks
		1		2		3	4	5	
		12 hour	24 hour	36 hour	48 hour	60 hour	84 hour	108 hour	
Allantoic	I	<10*	<10	40	640	1280	1280	-	
	II	<10	<10	20	20	-	320	-	
	III	<10	<10	20	<10	-	-	-	dead at 48 hours
	IV	<10	<10	20	80	-	-	1280	
Amniotic	I	<10	<10	<10	160	320	-	-	dead at 60 hours
	II	<10	<10	20	1280	-	-	-	

* Reciprocal of the titre of the H.A. test

ϕ Inoculum same as in expt. II; diluted at 10^{-2}

TABLE : III

The Developement of Haemagglutinin in Individual Eggs After Inoculation of Mumps Virus(ENDERS Strain)into the Allantoic Cavity

(EXPERIMENT : IV)

Time after inocula-tion	Egg no.	H.A. titre(reciprocal)							Geo. mean (log)
		10	20	40	80	160	320	640	
48 (hrs.)	1-5	0	0	nd					
72 (hrs)	1	2	0	nd					
	2	3	0	nd					-1.0 (appx)
	3	0	0	nd					
	4	0	0	nd					
	5	4	3	nd					
96 (hrs.)	1	4	4	4	4	2	0	0	
	2	4	4	4	2	0	0	0	
	3	4	4	4	4	2	0	0	-2.0
	4	4	4	4	4	2	0	0	
	5	4	4	4	4	1	0	0	
120 (hrs.)	1	4	4	4	4	4	2	0	
	2	4	4	4	4	3	1	0	
	3	4	4	4	2	0	0	0	-2.35
	4	4	4	4	4	4	1	0	
	5	4	4	4	4	3	1	0	
144 (hrs.)	1•	4	4	4	4	1	0	0	
	2	4	4	4	4	4	1	0	
	3•	4	4	4	2	1	0	0	-2.1
	4	4	4	4	4	4	2	1	
	5	4	4	4	4	4	1	0	

nd. = not tested at the particular dilution

• The allantoic fluid was slightly viscid probably due to albuminous material

TABLE : IV

The Appearance of Haemagglutinin in Allantoic Fluid After Intra-Allantoic Inoculation of the Mumps Virus (EXPERIMENT : V)

Time after inoc. (Hours)	Egg no.	H.A. titre(reciprocal)									Geo. mean (log)
		5	10	20	40	80	160	320	640	1280	
ϕ12	1,2	0	0	0	nd	..	..	..	..	..	-0.6▲
ϕ36	3,4	0	0	0	nd	..	..	..	..	..	-0.6▲
ϕ48	5,6	0	0	0	nd	..	..	..	..	..	-0.6▲
60	7	0	0	+	0	0	nd	..	..	..	-0.6▲
	8	+	0	0	0	0	nd	..	..	..	
72	9	4	4	4	2	0	0	0	0	0	-1.55
	10	4	4	4	3	1	0	0	0	0	
84	11	4	4	4	4	1	0	0	0	0	-1.9
	12	4	4	4	4	4	2	1	0	0	
96	13	4	4	4	4	2	+	0	0	0	-2.05
	14	4	4	4	4	2	2	1	0	0	
108	15	4	4	4	4	4	2	1	0	0	-2.15
120	16	4	4	4	4	4	4	4	2	1	-2.75
130	17	4	4	4	4	4	4	4	1	0	-2.5

ϕ At 12,36.48 hours all the 6 egg-fluids were tested individually and found negative.

▲ Calculated by assuming that a dilution less than 1:5 (say 1:4) could have been positive

nd: not tested

TABLE : IVa

The Haemagglutinin Content of the C.A.Membrane Suspensions

(EXPERIMENT : V)

Time after inoc. (hrs.)	Egg no.	Reciprocal of the final dilution of the suspension						
		4	8	16	32	64	128	256
12	1	*2	2	1	0	0	0	0
	2	1	±	0	0	0	0	0
36	3	0	0	0	0	0	0	0
	4	0	0	0	0	0	±	0
48	5	0	0	0	0	±	0	0
	6	0	0	0	0	0	0	0
60	7	0	0	0	0	0	±	0
	8	0	0	0	0	±	±	0
72	9	0	0	0	0	0	1	0
	10	0	0	0	0	0	0	0
84	11	0	0	0	2	0	0	0
	12	0	±	1	0	0	0	0
96	13	2	0	0	0	0	0	±
	14	1	0	0	0	0	±	0
108	15	3	2	2	0	0	0	0
120	16	4	4	3	2	±	0	0
132	17	4	4	4	4	2	0	0

*Indicate the degree of haemagglutination reaction.

TABLE : V (a)

Titration of the Infected Allantoic Fluids for 'Viral' Complement Fixing Antigen

(EXPERIMENT V)

Time after inoculation	Egg no.	Dilution of allantoic fl.(initial)					
		vs.Anti-serum				compl.contr.	
		1:2	1:4	1:8	1:16	1:2	1:4
12 hrs.	1&2	*2	3	4	4	2	4
36 ..	3&4	4	4	4	4	4	4
48 ..	5&6	4	4	4	4	4	4
60 ..	7&8	4	4	4	4	4	4
72 ..	9&10	4	4	4	4	4	4
84 ..	11&12	0	2	4	4	4	4
96 ..	13&14	0	0	4	4	4	4
108 ..	15	0	0	3	4	4	4
120 ..	16	0	0	0	3	4	4
130 ..	17	0	0	0	4	4	4

TABLE : V (b)

Titration of Infected C.A. Membrane Suspension for the Content of Complement Fixing Antigen('Membrane'Antigen)

(EXPERIMENT V)

Time after inoc. (hrs.)	Egg no.	Dilution of membrane susp.(initial)							
		vs. Anti-serum						comp.cont.	
		1:4	1:8	1:16	1:32	1:64	1:128	1:2	1:4
12	1&2	*4	4	4	4	4	4	4	4
36	3&4	4	4	4	4	4	4	4	4
48	5&6	4	4	4	4	4	4	4	4
60	7&8	3	4	4	4	4	4	4	4
72	9&10	1	3	4	4	4	4	4	4
84	11&12	0	2	3	4	4	4	4	4
96	13&14	0	0	1	4	4	4	4	4
108	15	0	0	0	0	4	4	4	4
120	16	0	0	0	3	4	4	4	4
130	17	0	0	0	0	2	4	4	4

*Degree of reaction: 0 (complete fixation)
4 (no ...)
2 (50 % ...)

TABLE : VI

The Developement of the Haemagglutinin in Allantoic Fluid After Inoculation of the Mumps Virus (EXPERIMENT VI)

Hours after inoc.	Egg no.	Haemagglutination titre (reciprocal of final dilution) 20	40	80	160	320	640	1280	2560	Geomet. mean (log)
INOCULUM 0	-	4	4	4	4	3	2	0	0	-2.8
12	1	0	0	0	0	0	±	-	-	
	2	0	0	0	0	0	0	-	-	<-1.3
24	1	0	0	0	1	±	2	-	-	
	2	0	0	0	0	<3	0	-	-	<-1,3
	3	0	0	0	0	0	±	-	-	
36	1	0	0	0	0	±	±	-	-	
	2	0	0	0	0	0	1	-	-	<-1.3
	3	0	0	0	±	0	0	-	-	
48	1	0	0	0	1	0	0	-	-	
	2	0	0	0	±	1	0	-	-	<-1.3
	3	0	0	2	0	0	0	-	-	
60	1	0	0	0	0	0	±	-	-	
	2	1	±	1	0	±	±	-	-	<-1.3
	3	1	±	0	0	±	0	-	-	
72	1	4	2	0	0	0	0	-	-	
	2	4	2	0	0	0	0	-	-	-1.5
	3	4	1	1	0	0	0	-	-	
84	1	4	4	4	2	±	0	-	-	
	2	4	4	4	3	0	0	-	-	-2.2
	3	4	4	3	2	0	0	-	-	
96	1	4	4	3	2	1	0	0	0	
	2	0	0	0	0	0	0	0	0	=2.2
	3	0	0	0	0	0	0	0	0	
108	1	4	4	4	4	3	0	0	0	
	2	4	4	4	3	<2	0	0	0	-2.4
	3	4	4	4	2	1	0	0	0	
120	1	4	4	4	4	2	±	0	0	
	2	4	4	4	4	2	1	0	0	-2.4
	3	4	4	4	4	1	1	0	0	
132	1	4	4	4	4	3	0	0	0	
	2	4	4	4	4	<2	0	0	0	-2.3
	3	4	4	4	2	0	0	0	0	
144	1	4	4	4	3	2	0	0	0	
	2	4	4	4	3	3	1	0	0	-2.5
	3	4	4	4	3	2	0	0	0	
168	1	4	4	4	4	1	±	±	0	
	2	4	4	4	4	3	1	0	0	-2.6
	3	4	4	4	4	4	4	2	0	
192	1	4	4	4	3	±	0	0	0	
	2	4	4	4	4	4	2	0	0	-2.6
	3	4	4	4	4	2	1	0	0	
216	1	4	4	4	4	4	3	1	0	
	2	4	4	4	4	3	2	±	0	-2.
	3	4	4	4	4	4	3	3	1	

Degree of reaction : 4 = complete aggltn.
0 = no ...
3 = more than 50% cells agglutinated
1 = less .. ..
2 = 50% cells agglutinated.

TABLE : VII

The Developement of the Complement Fixing Antigen in Allantoic Fluid After Inoculation of Mumps Virus (pooled samples) ... ExperimentVI.

Time after inoc. (hrs.)	Initial dilution of the pooled fluid (reciprocals) vs. anti-serum										
	2.5	5	10	20	30	40	60	80	100	120	160
12	4	4	4	4	4	4	-	-	-	-	-
24	4	4	4	4	4	4	-	-	-	-	-
48	4	4	4	4	4	4	-	-	-	-	-
72	0	4	4	4	4	4	-	-	-	-	-
96	0	0	0	2	4	4	4	4	4	-	-
120	0	0	0	0	1	3	4	4	4	4	4
144	0	0	0	0	0	0	1	2	<4	4	4
168	0	0	0	0	1	1	3	4	4	4	4
192	0	0	0	0	1	1	4	4	4	4	4
216	0	0	0	0	0	1	1	2	<3	3	4
Inoculum	0	0	0	3	4	4	4	4	4	4	4

Degree of reaction : 0= complete fixation
1= partial ...
2= 50 % ...
3= almost comlete haemolysis
4= complete haemolysis or no fixation.

TABLE : VIII

Titration of Pooled Allantoic Fluids for the Developement of Infective Property After the Inoculation of Mumps Virus

(EXPERIMENT VI)

Hours after inocul.	Final dilution of the pooled fluid							Calculated EID_{50} (per ml.)
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
0	4/4^^	4/4	3/4	3/4	1/4	0/4	0/4	$10^{-5.5}$
12	0/4	0/4	0/4	0/4	0/4	0/4	-	$<10^{-1}$
24	0/4	0/4	0/4	0/4	0/4	0/4	-	$<10^{-1}$
36	0/4	0/4	0/4	0/4	0/4	0/4	-	$<10^{-1}$
48	4/4	3/4	0/4	0/4	0/4	0/4	-	$10^{-3.3}$
60	4/4	4/4	1/4	0/3	0/4	0/4	-	$10^{-3.66}$
72	-	4/4	3/4	1/4	0/4	0/4	0/4	$10^{-4.5}$
84	-	4/4	4/4	3/4	0/4	0/4	0/4	$10^{-5.33}$
96	-	4/4	4/4	4/4	3/4	2/4	0/4	$10^{-6.56}$
108	-	0/4	0/4	0/4	0/4	0/4	0/4	$<10^{-2}$
120	-	4/4	4/4	0/4	0/4	0/4	0/4	$10^{-4.5}$
132	-	4/4	3/3	0/4	0/4	0/4	0/4	$10^{-4.5}$
144	-	4/4	4/4	3/3	3/4	1/4	0/4	$10^{-6.34}$
168	-	4/4	3/4	1/4	0/4	0/4	0/4	$10^{-4.6}$
192	-	4/4	1/4	0/4	0/4	0/4	0/4	$10^{-3.66}$
216	-	4/4	3/4	2/3	1/4	0/4	0/4	$10^{-5.23}$

^^ figure in the numerator = number of egg injected,
.. .. .. denominator= .. .. .. inoculated.

TABLE : IX

Titration for Haemagglutination and Haemolytic Activity of Mumps Virus (Enders strain) During Its Growth in Allantoic Cavity of Embryonated Eggs

Fluid Hours after inoculation	Age of Embryo (Days)	Test	Reciprocal of the initial dilution of allantoic fluid							
			5	10	20	40	80	160	320	640
12	8 1/2	H.A.	0	0	0	0	0	0	0	-
		H.Ly.	+.11	+.05	-.02	-.025	-.02	+.01	-.025	-
24	9	H.A.	0	0	0	0	0	0	0	-
		H.Ly.	-.065	-.055	-.065	-.045	-.04	-.015	-.025	-
36	9 1/2	H.A.	0	0	0	0	0	0	0	0
		H.Ly.	-.02	-.055	-.01	-.01	-0025	+.005	-.01	-.01
48	10	H.A.	0	0	0	0	0	0	0	0
		H.Ly.	-.09	-.085	-.05	-.025	-.05	-.05	-.05	-.025
60	10 1/2	H.A.	0	0	0	0	0	0	0	0
		H.Ly.	+.015	-.03	-.06	-.045	-.035	-.03	-.055	-.055
72	11	H.A.	2	0	0	0	0	0	0	0
		H.Ly.	0	+.075	??	0	-.025	-.025	-.025	-
84	11 1/2	H.A.	4	4	4	2	0	0	0	0
		H.Ly.	+.045	+.12	+.02	+.03	-.005	+.03	+.005	+.005
96	12	H.A.	4	4	4	2	0	0	0	0
		H.Ly.	+.005	+.015	+.160	0	-.045	-.045	-.050	-.005
108	12 1/2	H.A.	4	4	4	4	2	0	0	0
		H.Ly.	+.16	+.175	+.135	+.105	+.095	+.025	+.015	+.005
120	13	H.A.	4	4	4	4	2	0	0	0
		H.Ly.	+.35	+.425	+.375	+.225	+.115	+.075	+.040	+.045
132	13 1/2	H.A.	4	4	4	4	3	0	0	0
		H.Ly.	+.625	+.52	+.355	+.245	+.185	+.125	+.075	+.045
144	14	H.A.	4	4	4	4	4	1	0	0
		H.Ly.	+.288	+.358	+.27	+.21	+.163	+.105	+.09	+.042
168	15	H.A.	4	4	4	4	2	0	0	0
		H.Ly.	+.112	+.128	+.092	+.088	+.065	+.05	+.055	+.04
192	16	H.A.	4	4	4	4	1	0	0	0
		H.Ly.	+.145	+.248	+.178	+.042	+.025	+.015	+.032	0
216	17	H.A.	4	4	4	4	4	2	0	0
		H.Ly.	+.65	+.7	+.655	+.55	+.375	+.190	+.150	+.11
Normal allan. fluid.		H.A.	0	0	0	0	0	0	0	0
		H.Ly.	+.015	+.01	-.025	+.005	+.03	+.025	+.01	0
Infected Inoculum		H.A.	4	4	4	1	0	0	0	0
		H.Ly.	+.69	+.63	+.48	+.36	+.23	+.16	+.075	+.02

N.B. Haemagglutination test was carried out at 4°C with 2% fowl cell suspensio
Haemolytic activity expressed in terms of colorimeter reading (difference between direct reading and mean oreading of saline comtrol tubes)

TABLE : X

The Developement of Haemagglutinin in Eggs After Inoculation of the Same Amount of Mumps Virus (0.1 ml. of 10^{-3} dilution) into the Allantoic Cavity

Egg no.	H.A. titre of the allantoic fluid				
	100	200	400	800	1600
1	4	4	2	±	0
2	4	4	1	0	0
3	4	4	2	±	0
4	4	2	0	0	0
5	4	4	2	0	0
6	4	1	±	0	0
7	4	4	4	2	±
8	4	2	0	0	0
9	4	4	3	1	0
10	4	4	2	0	0
11	4	4	4	1	0
12	4	4	1	0	0
13	4	1	0	0	0
14	4	4	3	2	0
15	4	4	3	±	0

The fluids harvested after 5 1/2 days incubation.

TABLE : XII

The H.A. titre of Individual Allantoic fluids After Inoculation of Different Dilutions of Mumps Virus

Dilution	No. of eggs	Calculated titre (50% H.A.) (r e c i p r o c a l)	Geomt. mean (log)
10^{0}	4	200, 600, 800, 800,	-2.72
10^{-1}	5	400, 600, 800, 1200, 1600,	-2.91
10^{-2}	5	600, 800, 1200, 1200, 1600,	-3.00
10^{-3}	5	600, 1200, 1200, 1600, 1600,	-3.07
10^{-4}	4	400, 600, 800, 1000,	-2.78

TABLE : XI

The H.A.titre of Pooled Allantoic Fluids After the Inoculation of Different Dilutions of Mumps Virus

Expt.	Dilution	No.of egg	H.A. test					Remarks
			100	200	400	800	1600	
"A"	10^{0}	4	4	4	2	0	0	Fluids harvested after 5 days of incubation
	10^{-1}	5	4	4	4	1	0	
	10^{-2}	5	4	4	4	3	±	
	10^{-3}	5	4	4	4	4	1	
	10^{-4}	4	4	4	2	±	0	
"B"	10^{0}	4	4	2	±	0	0	Fluids harvested after 6 days of incubation
	10^{-1}	4	4	4	1	0	0	
	10^{-2}	4	4	4	4	2	0	
	10^{-3}	4	4	4	4	4	1	
	10^{-4}	3	4	3	±	0	0	

DISCUSSION

The present knowledge of the nature of development of the viruses in host tissue is mainly based on numerous observations on the multiplication of bacteriophage and of the influenza viruses. It is probable that their biological multiplication is fundamentally similar in nature. By analogy certain working hypothesis have been suggested to explain some of the experimental phenomenon. But, there is also no reason to believe that the evidence of the nature of the growth of one virus should in every case be applicable to elucidate that of another virus.

The results of the foregoing experiments throw some light on the nature of the development of mumps virus, after inoculation into the extra embryonic cavities of a growing egg. Although all the experiments have not been carried out under identical conditions, whenever they were repeated the results proved closely comparable.

From the available evidence it appears that the mumps virus multiplies at a relatively slow rate in the chick embryo and reaches a maximum titre in approximately 5 days. In this respect, it differs from other members of the Influenza-Mumps-Newcastle disease virus group, which grow at a much faster rate/

rate and reach maximum titres within 2 days.

With the above group of viruses it is generally believed that the haemagglutination test may be used as an index to measure the virus content. On that basis the growth of the mumps virus was determined in every experiment according to the haemagglutinin content of the embryonic fluids or the tissue. In one of the experiments the development of the haemagglutination activity was correlated with that of the infectivity, and the complement fixing and haemolytic properties.

In the results presented there is nothing to show that the activities of the mumps virus during its stages of development are due to anything other than the virus particles themselves. Although there is a general correlation and parallelism in the above activities certain discrepancies have also been noted. The difference between the results of the infectivity titration and that of the haemagglutination tests has been particularly emphasised because these two tests are commonly employed to determine the virus content of any preparation. In the light of the experimental findings and from the relevant discussions it will appear/

appear that the haemagglutination titre determinations may not reflect accurately the rate of mumps virus multiplication.

From the available evidence it appears that the mumps virus probably passes through the following stages of development.

(I) 12 hours after intra-allantoic inoculation the virus could not be detected in the allantoic fluid. It is obvious that a majority of the virus particles were adsorbed on the cells of the C.A. membrane and whatever was left in the allantoic fluid could not be detected at the minimum dilution used in the different tests.

It is likely that the phase of adsorption was much quicker and that it could be determined correctly if the allantoic fluids were examined at shorter intervals. However, even at 12 hours the haemagglutinins were demonstrated in the membrane. As indicated earlier this must have been due to the presence of the residual adsorbed virus.

(II) A lag phase: It begins very soon after the virus has adsorbed on the cells of the C.A. membrane and extends until there is an indication of the virus in the allantoic fluid. During this phase the virus can not be demonstrated in the membrane suspensions either/

either by the C.F. test or the H.A. test. The lag phase probably lasts for approximately 36 hours.

It became apparent that the time interval depended on the amount of the seed virus. With a large amount of the virus, that is, with less diluted inoculum the virus haemagglutinin was detected in the allantoic fluid even within 36 hours. On further dilution of the inoculum the haemagglutinin was evident between 66 and 72 hours. In the latter case, however, the virus could be recognised much earlier by infectivity titrations at 48 hours. A similar observation has been made by Ginsberg et al. (1948a), Ginsberg and Horsfall (1949a). The latter workers also found that this minimum time was 28 to 32 hours with a concentrated inoculum and about 96 hours with diluted seed virus. The slight difference is possibly due to the fact that these authors used a different strain (Habel strain) of the mumps virus.

(III) The lag period is followed by another phase when there is liberation or excretion of the virus particles from the infected cells into the allantoic fluid. It is however continuous with another phase of adsorption and a repetition of the cycles, because it is natural to assume that the liberated virus particles/

particles would infect further batches of uninfected cells.

The presence of the infective property in samples of allantoic fluids which did not show haemagglutination, complement fixing antigen or the haemolysin is noteworthy. This finding is contrary to what is usually known to characterise the influenza viruses or the bacteriophages. Thus Gard and Von Magnus (1947), Hoyle (1948), Henle (1949b) have postulated that immature non-infective forms of influenza virus is formed during the early stages of intracellular multiplication. This form shows haemagglutination and complement fixing properties in spite of its lack of infectivity. However, an opposite phenomenon has also been observed recently by Henle and Henle (1949c) and Blumenthal et al. (1950), who under certain conditions could demonstrate a rise of the infectivity before a rise of haemagglutinin.

It appeared from the present data that virus titres (E.I.D.50) of the order of approximately $10^{-4.5}$ per ml. are necessary before the haemagglutinin can be demonstrated. In the serial titrations of the allantoic fluids, the haemagglutinin was detected about 36 hours after the infective property reached a significant titre. This is supported by similar/

similar observations made by Ginsberg et al. (1948a). The authors however failed to detect an earlier rise of the infectivity titre because they did not test the serial samples at shorter intervals.

From the above findings it is tempting to believe that the newly formed mumps virus is endowed with infectivity and that it is only during the extra cellular phase of maturity that other properties are manifested. It appears pertinent to point out that during the whole period when the allantoic fluid did not show the presence of the haemagglutinin or the complement fixing antigen, the membrane suspensions were also found to be negative.

However, it is possible that the apparent discrepancy between the infectivity and H.A. titrations during the early phase of the growth of the virus may be due to the following factors:

(1) The infectivity titration is obviously far more sensitive and can reflect smaller variation in the concentration of the virus. It is therefore possible that until the concentration reaches certain threshold the haemagglutinins will not be detected. The findings probably support this theory.

(2) The haemagglutination (H.A.) may be inhibited to a variable extent by a large number of biological/

biological materials, including the components present in the embryonic fluid and tissue extract (Anderson, 1949). The virus may be bound by the allantoic fluid inhibitor, so that the H.A. is masked. The infectivity of this bound virus is not impaired (Hardy and Horsfall, 1948).

When a bigger dose of the virus is introduced into the allantoic cavity, there is an infection of a large number of cells of the C.A. membrane. At the end of about 36 hours, when the first cycle is completed, there is a release of large number of virus particles so that their concentration reaches the optimal threshold. Under such condition, a positive H.A. reaction is obtained (experiment III). On the other hand, with a smaller dose of the virus (diluted inoculum) the number of infected cells will also be less. On the completion of the first cycle of growth, the concentration of the released virus may not reach the threshold to cause H.A. either due to actual diminution in number or due to the inhibitory factors present in the allantoic fluid. The newly formed virus is soon 'lost' by being adsorbed on the uninfected cells of the C.A. membrane and another cycle of growth is initiated.

During/

During this phase the residual virus left free in the allantoic fluid is responsible for maintaining the virus titre. If the rates of liberation of the newly formed virus and their adsorption on uninfected cells are somewhat balanced, a state of equilibrium is reached for some time. Under this condition the concentration of the virus may remain constant and no significant increase will be apparent either by the infectivity titre (experiment VI) or the H.A. titre (experiment II). Of course, it is not always possible to demonstrate this phase of equilibrium unless the samples are examined at shorter intervals.

A similar observation may be made from the experimental results published by Ginsberg and Horsfall (1949). After an inoculation of diluted virus-seed positive H.A. was obtained at 96 hours. On the other hand with undiluted virus seed, H.A. was positive at 28 hours and a concentration plateau appeared between 32 to 36 hours. It persisted for about 12 hours after which there was another rise of the H.A. titre.

If the above assumption that one cycle of growth lasts for a period of about 36 hours is correct, one should expect to find that at or before 72 hours there/

there will be an appreciable amount of haemagglutinin even after the inoculation of diluted virus. The experimental results have shown that the above contention is true. In fact, at about 72 hours positive results were obtained with the H.A., C.F. and haemolysis tests.

Additional experimental evidence in support of this conclusion is shown by the decline in the infectivity titre after 96 hours in experiment VI. The decline in titre and then a plateau of concentration, together lasting for about 36 to 48 hours approximate the period of a single cycle as mentioned earlier. The H.A. titre also showed a tendency to decline during this interval.

It therefore seems probable that the multiplication of mumps virus occurs in a succession of intracellular cycles and if the inoculum contains small amount of virus, the latter may not be demonstrable until the second cycle is completed. Henle et al. (1947a), Hoyle (1948), Blumenthal et al. (1950) and other workers have observed similar phenomenon during the multiplication of the influenza virus in eggs. It is, however, obvious that a more accurate determination of the growth cycle can only/

only be made by a study of the "one step growth curve" (Ellis and Delbrück, 1939; Delbrück and Luria, 1942; Henle and Rosenberg, 1949 a, b).

It should be pointed out in this connection that contrary to the observations made with the influenza viruses, the haemagglutinin and the complement fixing antigen contents of the membranes did not show any increase during the initial period of 60 hours after inoculation. A rise was evident only at the time when the haemagglutinin content of the allantoic fluid also showed a tendency to increase. It is not possible to say whether the membrane suspensions were infective during this early phase, as no infectivity titrations were performed. Afterwards, there was uniform increase of both the haemagglutinin and the complement fixing antigen contents of the allantoic fluid and C.A. membranes.

It is worthwhile to include in this section the observations made during the routine maintenance of the Enders strain of mumps virus. Before the variations of the infectivity titre or the effect of dilution of the inoculum could be recognised, the virus was maintained by inoculating eggs at 10^{0} to 10^{-1} dilutions of the pooled allantoic fluid. It/

It was found after 8 to 10 egg passages that the pooled fluid generally showed lower titres of the H.A. and C.F. tests. Whereas in the beginning the virus showed infectivity of 10^{-5} to 10^{-6}, in two subsequent passages the infectivity was apparent only at 10^{-3} to 10^{-4} dilution.

On the basis of the results of the experiments described in this section it seems reasonable to consider the possibility that (i) due to early discharge of the newly formed virus at about 36 hours, the repeated cycles permitted an accumulation of some virus which became inactivated towards the end of 5 days; (ii) due to a periodicity of the rise and fall of the infective titre some of the pooled fluids contained increasing amounts of inactive virus particles. It is possible that the presence of the latter was responsible for an effect similar to the interference phenomenon between active and inactive influenza viruses (Henle, 1947).

From the practical point of view all these findings seem to emphasise the need for a proper selection of the seed virus. Moreover, it is clearly apparent that to obtain virus pools for the preparation of diagnostic antigens, for the seed virus, or for other studies, there is a need for careful selection of the period after which the eggs should be harvested.

CHAPTER V

The Isolation of the Virus from Cases of Mumps and the Observations on the Adaptation of Newly Isolated Strains to grow inside the Allantoic Cavity of Embryonated Eggs.

Isolation of the Mumps Virus.

In the past with few exceptions, the virus has been isolated from the saliva of patients with parotitis. Its isolation from the spinal fluid of patients with mumps meningo - encephalitis (Swan and Mawson, 1943; Henle and McDougall, 1947), from the testicular tissue (Friedewald, 1949) and from blood (Kilham et al.,1949) has also been possible. The virus is said to have been isolated as well from the parotid gland and possibly either from the pancreas or the ovary at the post-mortem of a patient suffering from an obscure symptom with parotitis.

The isolation of the virus from saliva is possible only when it has been collected in the early stages of the disease. Under experimental conditions after the virus has been sprayed inside the oral cavity of human volunteers or after close contact with infected patients, the presence of virus can be detected in saliva even before the onset of the parotitis, i.e. during the incubation of the disease (Henle et al., 1951b).

Collection and preservation of Saliva:

Patients ill with parotitis during the acute inflammatory stage of the disease were selected for the investigation. The oral cavities of these patients were washed with sterile distilled water or saline before the collection of saliva. Sterile

cotton-wool plugs were placed opposite the opening of the Stensen's duct on the affected side or sides. The patients were asked to hold them in position by closing the mouth and applying pressure with the cheeks. After about half an hour the cotton-wool plugs were removed and placed inside sterile glass containers. Any excess of salivary secretion which the patients could furnish by squeezing the cheeks were also collected. Fresh cotton-wool plugs were inserted again and the process repeated once or twice during the period of one to two hours over which the salivary secretion was collected. Saliva with the soaked cotton-wool plugs were quickly frozen at the bedside over solid CO_2 - ice ($-36^{\circ}C$) contained in a thermos flask and carried to the laboratory. They were stored at the same temperature until eggs were available for inoculation. In no instances had the saliva to be stored for more than a period of 7 days before the inoculation.

Preparation of the inoculum:

The cotton wool plugs and the frozen saliva were soaked in 2 to 5 ml. of sterile buffered saline (p^H.7.2) containing salts of sodium penicillin G and streptomycin sulphate in concentrations of 250 and 2500 units per ml. respectively. The plugs were wrung out several times in saline with the help of two sterile forceps. The emulsified saliva was spun/

spun in an angle-head centrifuge at 2,500 r.p.m. for 15 to 20 minutes to deposit large coarse particles and presumably a portion of the bacteria were also eliminated. Eggs were inoculated with the supernatant fluid without any further treatment.

Inoculation of eggs, harvesting and further passages:

Usually 12 to 16 eggs were used for each specimen through the amniotic route, using a dose of 0.1 to 0.15 ml. of the supernatant salivary emulsion. The methods adopted for the inoculation have been already described. The eggs which were incubated for 5 days at 35°C after the inoculation were harvested for the amniotic fluids and the amniotic membranes. These were stored at 4°C overnight. The amniotic fluids were each tested for the presence of virus by H.A. test using the erythrocytes of the fowl, the guinea-pig and the group 'O' cells. They were also tested for sterility on blood agar plates. The fluids which were found sterile and gave positive H.A. reactions were pooled and repassed amniotically into batches of 8 to 12 eggs.

The sterile fluids with negative H.A. reaction were pooled. The corresponding amniotic membranes were also pooled and emulsified in a Ten - Broeck tube using the amniotic fluid as diluent. The suspension was centrifuged briefly to throw down the coarse particles and the supernatant used for

re-passage in eggs as above. At least three passages were made in eggs before any specimen of saliva was considered negative for the presence of mumps virus. Identification of the haemagglutinating agent in the positive cases of isolation was then performed and the results are embodied in another section of this thesis.

Collection of blood and preparation of the inoculum:

Two different methods were used for the collection of blood by veinipuncture. In the first method, as described by Kilham et al. (1949), about 5 ml. of blood was mixed with sterile sodium citrate solution to give a final concentration of 0·5%. The plasma and the cells were separated by centrifugation and preserved at -36°C. until inoculation. The plasma was injected into the eggs without further dilution. The cells were resuspended in buffered saline to give a 20 to 30% concentration before the inoculation into the eggs.

In the second method, about 5 ml. of the blood was allowed to clot. Most of the serum was removed and the rest together with the blood clot were crushed by the help of a pestle and mortar. Powdered glass was used as an abrasive. About 2·5 ml. of saline was added to resuspend the crushed blood clot and

the supernatant fluid after light speed centrifugation was injected into the eggs.

The dose or the inoculum and the handling of the eggs after inoculation were the same as described for the salivary emulsion.

RESULTS

A. Isolation of the virus from Saliva

An attempt was made to isolate the virus from saliva of five patients. In two cases only the attempt was successful. These two strains of the virus have been designated as the JAMES and the PAUL strains. The following table (1) summarises the findings.

No. Patients	Day of the disease	Specimen of saliva	First positive embryo passage	Remarks
1 James Wh.	2nd 4th	1st 2nd	- 1st	*Negative
2 Margaret Yu.	4th	1st	-	Negative
3 Paul Sw.	1st 2nd	1st 2nd	- 1st	Negative
4 Richard Sw.	2nd 3rd 5th	1st 2nd 3rd	- - -	Negative Do. Do.
5 Catherine Hu.	3rd	1st	-	Negative

With every specimen of salivary emulsion it was observed that a variable number of the inoculated embryos died within 24 to 72 hours. In occasional eggs the inoculated amniotic fluid showed contaminating organisms. In the majority of others, however, it was not possible to account for the death on basis/

basis of bacterial infections or due to the trauma during inoculation.

Some of the negative specimens of saliva were repeatedly inoculated into different batches of eggs. It was found that the mortality rate amongst the embryos decreased with the dilution of the inoculum. It was also observed that there were fewer deaths amongst the 9 or 10 day old embryos than those in the younger ones. It is possible that certain factors present in saliva are toxic to the young embryos. In subsequent passages there was rarely any death of the inoculated embryo.

In both instances of positive isolation, the virus was demonstrated in the first amniotic passage by H.A. tests. During this primary isolation only a small proportion of the amniotic fluids gave positive haemagglutination. Thus, with the James strain only 3 out of 12 eggs and with the Paul strain 2 out of 6 eggs showed the presence of virus. Even in the 2nd passages about half the number of eggs showed the growth of the virus although all of them were inoculated with positive fluid. It was only from the 3rd amniotic passage and thereafter that the eggs were regularly infected.

Burnet and Bull (1943) found strains of influenza virus, during the first passage in amniotic cavity of eggs, had little capacity to agglutinate fowls cells, though they could agglutinate those of man and guinea pig to an appreciable degree. A similar/

similar phenomenon could not be observed with the newly isolated strains of mumps virus. In the first four amniotic passages the positive fluids showed equivalent H.A. titres with both the fowl and the guinea pig cells. As compared with these, the titres obtained with group 'O' human cells were usually lower.

However, during the adaptation of the James strain to grow in allantoic cavity certain peculiarities of haemagglutination were noticed. The allantoic fluids from the second egg-passage were tested with different types of red cells. It was found that although five fluids showed positive haemagglutination with fowl and guinea pig cells, the H.A. test was positive with only two fluids when group 'O' human cells were used.

B. Isolation of the virus from blood

From each of the five patients shown in table I, the venous blood was collected on the day when the first sample of saliva was also obtained. A citrated specimen of blood was collected from James wh. The samples from others consisted of whole blood.

9 day old embryos were inoculated with suitably prepared materials. It was not possible to isolate the virus from the blood of any of these cases.

Adaptation of the newly isolated strains to grow inside the allantoic cavity

(a) James strain:

The positive fluid (pooled) from second amniotic passage was diluted and injected into the allantoic cavities of a fresh batch of eggs. None of these eggs was found infected. With the positive fluid from fourth amniotic passage, about 50% of the allantoically inoculated eggs showed positive haemagglutination. Irregular results were obtained in the second allantoic passage, but from the third passage onwards all the eggs became infected.

Except in an earlier passage (see before) the virus grown in the allantoic cavity has always agglutinated the fowl, the guinea pig and the group 'O' human cells. It has also been observed that the infectivity (E.I.50 dose) of the virus increased with repeated allantoic passages ($10^{-4.5}$ in 3rd passage and 10^{-6} in 7th passage).

(b) Paul strain:

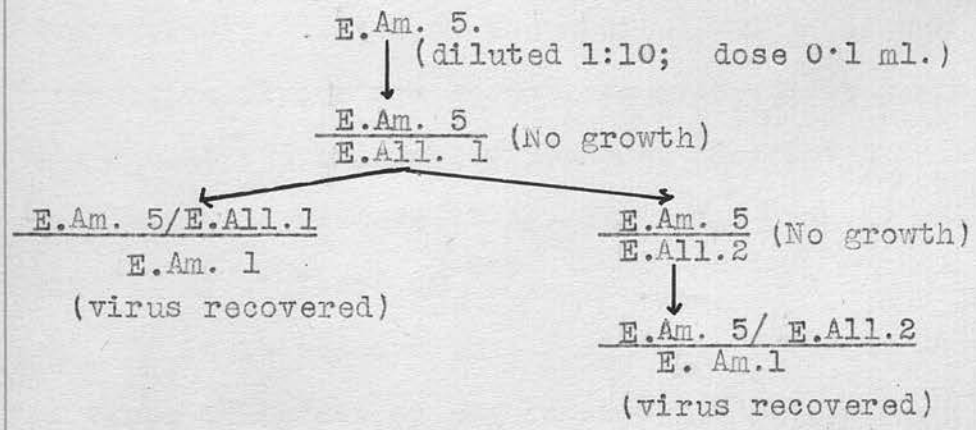
In spite of several attempts it has not been possible to adapt this strain in the allantoic cavity. The virus from the third, fifth and seventh amniotic passes was inoculated into the allantoic cavity. At least three blind allantoic passages were carried out on each occasion, using undiluted fluids. The results were uniformly negative by H.A. tests.

However,/

However, two experiments carried out in this connection serve to indicate that there was probably some growth of the virus inside the allantoic cavity, although it could not be detected by the H.A. test.

Experiment I:

The virus from 5th amniotic passage (E.Am. 5) was inoculated into the allantoic cavity (E.All. 1); another blind passage (E. All. 2) was made after appropriate interval. The allantoic fluids in both cases were negative by the H.A. test. However, when these fluids were again inoculated into the amniotic cavity, the virus could be recovered. This is shown schematically as follows:



In this experiment, although the passages were made with undiluted egg-fluids, it is doubtful whether the recovery of the virus after the second allantoic passage was due to the persistence of the original virus. The latter was diluted at least 1:500,000 when it was present in the eggs of E.Am. 5/E. All.2/E. Am.1. Moreover, during the whole period/

period of this experiment it was subjected to incubation at 35°C for 10 days and storage at 4°C for 6 to 7 days during the intervals.

Experiment II

The above experiment was repeated with the virus from seventh amniotic passage (E. Am. 7) and identical results were obtained. To obviate the dilution factor, for each passage the inoculum was dilated 1:50 and a constant dose of 0·1 ml. was injected into each egg.

The infectivity titre (E.I.D. 50/ml.) of the virus was determined as $10^{-5·2}$. The volumes of the allantoic and amniotic fluids of the 14 day eggs were found to vary between 4·5 to 5·5 ml. and 1·5 to 2 ml. respectively. Even if the minimum values of the respective fluids are taken into account, it may be shown that the original virus when inoculated in the last amniotic passage (E.Am. 7/E All.2/E Am.1) was diluted to at least $10^{-9·5}$.

If it is assumed that a minimum effective dose of virus to infect an egg is about 10 E.I.D. 50, the difference between the titre of the original virus and the final dilution as shown above can only be explained on the basis of the multiplication of the virus inside allantoic cavity.

CHAPTER VI

SECTION (A): The Serological Diagnosis of Mumps.

SECTION (B): Naturally Occurring Antibody in Human Serum against Mumps Virus.

THE IMMUNITY REACTIONS IN HUMAN BEINGS FOLLOWING MUMPS

The present study was undertaken to follow the development of antibodies after mumps infection, in so far as they were related to a serological diagnosis. In an effort to determine the fate of these antibodies long after the disease process was over, the study was extended to examinations of normal human sera. It was thought that a retrospect diagnosis of mumps or evidence of the presence of antibodies in these sera may be helpful in differentiating susceptible from immune persons. The need for such a differentiation is obvious in the formulation of public health programmes especially from the preventive aspect.

The scope, nature and the results of these investigations have been outlined in two different sections:

A. Serological diagnosis of mumps.

B. Naturally occurring antibody in human serum against mumps virus.

Section (A).

The Serological Diagnosis of Mumps

Until 10 years ago, the diagnosis of mumps was mainly based on clinical manifestations. Although/

Although neutralising antibodies against the mumps virus were demonstrated in convalescent sera by Johnson and Goodpasture as early as 1934, it was only in 1942 that an 'in-vitro' complement fixation test was described by Enders and Cohen. The test employed mumps infected monkey parotid gland emulsion as the antigen. The importance of serological diagnosis in obscure cases of virus meningoencephalitis without swelling of the parotid glands was realised from successful investigations of Kane and Enders (1945). By means of complement fixation tests they were able to demonstrate that 50% of these cases were due to infection by mumps virus.

When the mumps virus could be adapted to grow in the developing chick embryo, more knowledge was gained about its properties. Several laboratory tests were devised to demonstrate the antibody after mumps infection with the egg-grown virus as antigen. The different techniques which have been described in the literature for routine serological diagnosis are:

1. the haemagglutination - inhibition (H.I.) test,
2. the complement fixation (C.F.) test, and
3. the Burnet test, or the agglutination by specific /

specific mumps antibodies of human red cells sensitised with the mumps antigen.

A brief outline of these tests and their relative diagnostic value as observed by different workers has been included in ChapterI(C). It is apparent from these earlier observations that the complement fixation test using viral antigen is regarded as the most reliable method to demonstrate the antibodies in convalescent sera. The opinion is still divided as to which of these tests is helpful for the diagnosis especially in the early stage of mumps infection.

Henle and her co-workers contend that the use of 'Soluble' antigen for complement fixation tests definitely increases the chance of an early diagnosis. While their findings have received support from Rice (1949) and Oldfelt (1949), there are other workers who describe the test as unsatisfactory not only for the early diagnosis but even during the later stages of the disease. It is noteworthy that the latter group of workers prepared their 'soluble antigens' either from a strain of mumps different from that used by Henle et al., or by methyl alcohol precipitation. It is not known whether the use of a different strain of virus could/

could modify the results of the test. It is also possible that some labile factor in the antigen which has greater affinity for the corresponding anti-S antibody was removed due to alcohol treatment and caused discrepancy in the results. Besides, the technique of performing the complement fixation tests were not strictly comparable to those employed by Henle and her co-workers.

Leymaster and Ward (1948) have described an 'in VIVO' neutralisation test for measuring the antibody in mumps immune sera. The test has not been used so far in measuring the antibody content during the active stage of mumps infection. Little is known about the nature of this antibody and its usefulness in the serological diagnosis of mumps.

The present investigations were undertaken in order to obtain information on the relative diagnostic value of the soluble and viral complement fixing antigens. The development of the complement fixing antibodies has been correlated with that of the haemagglutination - inhibiting and neutralising antibodies. In addition, the experience in titrating some of the mumps sera by the Burnet test has also been outlined.

For/

For a proper analysis of the diagnostic procedures the antibody titrations have been carried out, mainly, with the sera from typical cases of mumps parotitis. A limited number of sera from patients with unusual manifestations of the infection were also examined.

The results of the immunity reactions are described in the following pages. Sera from several mumps patients were tested for the content of haemagglutination - inhibiting antibody against different strains of the mumps virus. These results are also included in this section.

Materials and Methods

Patients: The present material consisted of 31 patients belonging to different age groups. About two-thirds of them were 12 years old or younger. 26 patients were diagnosed clinically as uncomplicated cases of mumps and manifested mild to moderate inflammation of either or both the parotid glands. Nine of these also showed involvement of the submandibular gland and one patient presented swelling of the sublingual gland. There were three patients who presented symptoms of encephalitis or meningism without accompanying involvement of the salivary glands; in these patients the participation of mumps virus was suspected because of previous/

previous contacts. Two patients had swellings of submandibular and sublingual glands only; no definite history of contact with mumps cases could be obtained from them. Twenty-six of these patients were in the City Fever Hospital, Edinburgh and were resident either in the town or in the neighbouring districts. There was one patient from the Infectious Diseases Hospital, Glasgow. Other patients were not hospitalised. Two of them were from Hawick and two from Edinburgh.

Sera:

A total of 66 venous blood samples were obtained from 31 individuals. Single specimens were collected from seven patients. From 24 patients two or more blood samples were obtained. Initial specimens were collected soon after the onset of the parotitis or illness and the second samples were obtained at least after five days. From 26 patients the first sample could be collected on or before eight days after the onset of the disease. The sera were separated aseptically from blood clots as soon as possible after the collection. They were preserved with an antibiotic mixture and stored at -36°C or -76°C by methods previously described.

Serological/

Serological tests:

The techniques of performing the H.I., C.F. and neutralisation tests have been described in Chapter ..II.. All the sera were tested against antigens prepared from the Enders strain of virus, unless stated otherwise. Some of the sera were also titrated for the H.I. antibody against James and Paul strains. The methods of obtaining virus pools and the standardisation of the antigens have been described previously.

The titrations for H.I. and C.F. antibodies were carried out with two batches of antigens. The tests were spread over several days. The antigens which were standardised previously gave the same end points with a known rabbit antiserum within the limits of experimental error. The antigens were stored at -36°C throughout the period of the titrations. At the beginning, for the complement fixation tests 'normal' viral and soluble antigens were included as controls. Since none of the test sera showed any evidence of the non-specific fixation, during subsequent titrations the control antigens were not usually used. All titres are expressed as the reciprocal of initial serum dilution for C.F. tests and/

and that of the final dilution for the H.I. tests.

The titrations for the neutralising antibody were made against a single pool of allantoic fluid infected with Enders strain (E.I.D.50:$10^{-4\cdot8}$). Small amounts of the infected fluid, dispensed in glass ampoules, were stored at -76°C throughout the period of performing all the tests. Each serum was tested against 100 E.I.D.50 of the virus. Smaller numbers of eggs (two to four) were used for each serum dilution. As explained in Chapters ....II....(general methods) and ....III..(on standardisation of antigens), the end points have been calculated on the basis of 50% protection at a particular serum dilution. The values are expressed as the reciprocal of the final serum dilution

All the specimens of sera from one individual were tested simultaneously in each test. The protocols of these titrations are given in appendix ...(C) . The significant end points are shown in tables included in this section. The degree of reaction in the tube showing the end point by a particular test (H.I. or C.F.) is shown in brackets. For calculation or graphical representation, the titres have been converted to their negative log values. The geometric mean of different titres has been taken as the average titre.

Experimental/

Experimental and Results

All the 66 sera were tested by the H.I. and C.F. tests. Neutralisation tests were performed with 62 sera (from 29 patients): four sera were considered unsuitable because of gross bacterial contamination.

Reproducibility:

(a) The H.I. tests with all the sera were made in two batches. The titres of the known 'positive control' and four of the sera repeated on the second day showed a variation of one tube (two fold) in only one case.

(b) All the C.F. tests were completed in three batches. Titrations against viral and soluble antigens were made on the same day. A known convalescent serum containing high titre of both the anti-V and anti-S was included as control. On the 2nd and 3rd day of the tests two sera tested on the first day were re-tested along with the control. In only two out of these six repeat titrations a variation of one tube (two fold) was noted.

(c) The neutralisation tests were performed over a period of about two months. Titrations were repeated on two occasions. The significance of the variation has been discussed in Chapter III (on/

(on standardisation of this test). The test virus which originally showed an infectivity titre of $10^{-4.8}$ (EID50) was still found to be infective at 10^{-4} after 6 weeks of storage at -76°C.

The results of the above tests are given in tables I to IV. Table..I... shows the antibody levels in individual sera collected from each patient on different days after the onset of the disease. The maximum fold rise in titre between the first and the subsequent samples collected from each patient are also shown in the same table. The number of sera collected on any particular day after the onset are shown in table...II...The table also shows the variations in the titres of different sera collected on the same day. The same is represented graphically in figure ...1... a to d (as template distribution), calculated in terms of negative log values of individual titres (log units). The average (geometric mean) titre for any particular day is also shown in the same figures and its actual value in log unit or reciprocal is given in table ....III..

Analysis of the results:

1). Haemagglutination - inhibiting antibody:

It is apparent from the above results (table .II..) that the H.I. antibody level within the first three days is comparatively low, the titres not exceeding 1 in 32. There is a slow rise in the antibody titre till about 7-8 days, although individual samples of sera may be seen with a titre of 1 in 256. It is noteworthy that out of 19 sera collected within five days after onset all except one showed a titre of 1 in 64 or less. It is reasonable to consider all these patients as susceptible persons; accordingly, it may be said that in the early stages of the disease (within four or five days) titres above 1 in 64 are suggestive of infection. These findings are in agreement with those of Robbins et al. (1949).

There is a sharp rise in titre between 8 to 12 days and as it appears from figure 1 (a) the maximum level is probably reached at about this period. In none of the sera collected after 10 days a titre less than 1 in 128 was evident. An average titre of above 1 in 400 (table ,III..) is maintained at least a month following the infection. Afterwards, the antibody level has a tendency to fall gradually. In ten individual sera which were collected from these patients between 1 to 18 months after onset,

a/

a majority showed the titre of 1 in 256 and none of them were found to be less than 1 in 128.

The antibody has been found to develop regularly in all the convalescent sera. In paired sera from 21 patients, where the first samples were collected on or before eight days, more than 4-fold increase in titre has been observed in every case with only one exception. The first sample in this particular pair showed a titre of 1 in 256 on the eighth day and showed a maximum titre of 1 in 512 on both 19th and 26th day (see table ..V.. and fig. ..7..). The average rise in titre is eight times or more when acute samples of sera are obtained within four to five days of the onset. A maximum fold-rise of 32 times has been seen in four cases.

2). Complement - fixing antibody:

The antibody against 'Soluble' antigen (Anti-S antibody) is detected very early during the infection. Even within the first two days it is possible to find titres as high as 1 in 16 in occasional samples. At four to five days significant titres were obtained in eight out of ten sera (table .II.). The antibody reaches a maximum level between nine to ten days and shows a tendency to decrease from 17 or 21 days onwards (figs 2. & 3.).

The/

The downward tendency of the curve in fig..3.... (or the reduced height of the column in fig..2...) at 11-12 days was due to inclusion of a serum (titre at less than 1 in 4) from an individual (I.Ba.) who failed to show a rise of the anti-S antibody. The majority of sera obtained during the course of the illness or during the early convalescence show titres ranging from 1 in 8 to 1 in 32. In no case a titre above 1 in 64 has been observed. Five samples collected from some of the patients between 3 to 18 months showed titres of 1 : 8 in one case and 1 : 4 in four cases. A template distribution of the titre of individual serum is shown in fig..1..(c).

Paired samples of sera were examined from 24 patients. In 21 cases the initial sample of serum was obtained within eight days after the onset of the disease. With these sera, four fold or more rise in titre was seen in 10 cases, 2 fold rise in 7 cases and there was no rise in titre in four cases (fig..7..... and table ...V....). The low 'fold-rise' or the absence of rise in titre in these 11 cases does not necessarily indicate that there was no rise in the antibody content. In fact, except in two instances the second samples of sera had titres of 1 in 8 or above (3 sera at 1:8; 3 sera at 1:16; 2 sera at 1:32 and 1 serum at 1:64). Of the two exceptional sera, in one there was a rise/

rise from 1:2 to 1:4 and in the other both the second and the initial samples showed titres of less than 1:4. These are considered as evidence of negative response.

It will therefore be observed, that the anti-S titre may reach a significant titre within eight days so that in subsequent samples of sera, it is not always possible to demonstrate - the 4-fold rise in titre - generally accepted as a criterion of significant rise of antibody. The usual low titre of the antibody even in convalescent serum is also responsible for this difference. In three patients where the initial samples were obtained after eight days (at 11, 11 and 14 days), the same low increase in titre was also evident (table .VI..). Thus out of 26 patients from whom convalescent sera were collected (after eight days) only two (I.Ba. and J. Dou.) failed to show the presence of the anti-S antibody in significant amount.

The antibody against 'viral' antigen (Anti-V antibody) is not detected as early as the anti-S antibody. Although an individual serum may show a significant titre within five to six days, in the majority (18 out of 22) they are either negative or show only the minimum significant titre (table .II..). From six days onwards there is a rapid increase of the/

the antibody, so that after 13 days high levels are consistently obtained. The maximum level is probably reached slightly later at about 17 to 21 days. From few of the samples obtained after this period it appears that the titre is well maintained till about 40 to 42 days after which there is gradual decline. At the end of nine months or even at 18 months it is not unusual to obtain titres of 1:8 or 1:32. The template distribution of the titre of each serum is shown in fig. 1.(d).

With paired samples of sera, where the initial serum was collected before eight days, a four-fold or greater rise in titre was observed in all cases (fig. 7. and table .V...). The majority of the sera showed 8 to 16 fold rise in titre and in one case even a 128-fold increase was observed. Practically all the 26 samples of convalescent sera showed a high level of the antibody. A titre as high as 1:512 was observed in one of the cases (J.Ja.).

Comparison between Anti-S and Anti-V antibodies:

The relationship between these two complement-fixing antibodies is best shown in fig... 4...

The/

The C.F. titre of each of the 66 test sera has been plotted in such a way that the figure in the abscissa represents its Anti-V value and that in the ordinate represents the Anti-S value. The line passing through zero at an angle of 45° differentiates the sera with higher Anti-S titres (upper left) from those with higher Anti-V titres (lower right). Each dot represents a particular serum. When placed on the 'slant-line' it indicates that the titres of Anti-S and Anti-V are identical. It will be found that of 23 sera collected within first seven days of the disease, only in one instance the titre of the Anti-V was higher than that of the Anti-S. A few may show identical titres, but the majority gives higher values of the Anti-S. In the second week, more sera than before show higher Anti-V and the general level of both the antibodies are much higher. It is only in the third week or after that most of the sera give higher values of Anti-V.

It will also be apparent from the earlier analysis of the results that, whereas, the Anti-S reaches a maximum titre between 9 to 10 days the Anti-V does not show the maximum rise till about 19-20 days. For this reason, unless the first sample/

sample of serum is collected very early in the disease, subsequent samples may fail to show a rise in titre and the findings are liable to be misinterpreted. Later, however, the fold rise of Anti-V titre is very high (fig..6..). In general, higher titres for the Anti-V are obtained after 13 to 14 days and specially after the 3rd week, when the level of the Anti-S has a tendency to decline. The Anti-V is also more consistently detected than the Anti-S and persists at well above the significant level long after the infection. In no case has there been a failure of rise of the Anti-V antibody. Only in one patient (S.Woo.) the immunogenic response seems to have been delayed.

3). Neutralising antibody:

The titre of the neutralising antibody in different sera (template distribution) is shown in fig..1..(b). 62 sera were tested altogether. Of these six were single specimens and the rest were obtained from 23 patients. Paired sera from 17 patients and three to four sera from six patients were examined (table ..IV..).

It will be found from the fig..1..(b) and table ..II.... that the antibody level in majority of the acute/

acute stage sera does not exceed 1:16 within the first four days. After five or six days there is a rapid rise of the antibody so that, on an average, the maximum level is reached within 13 to 14 days. It is always possible to demonstrate increased level of the antibody after nine days. A high level is probably maintained for about three months. In few of the samples collected after this period a level higher than the normal was always detected.

It will be evident from table...II... that in acute stage sera 19 out of 24 had a titre of 1:32 or less. When the rise of titre in paired samples of sera are considered, it will be found that in every case, except one, there was a rise of eight-fold or more (table...V...).

4. Burnet's test:

The difficulties encountered in preparing modified red cells for the agglutination test has been mentioned in Chapter...III... Repeated attempts to prepare the sensitised cells after treatment with freshly harvested allantoic fluid infected with the Enders strain of virus were unsatisfactory. With one of the newly isolated strains(James) satisfactory batches of sensitised cells were prepared and they were found to conform to the'receptor gradient' described by Burnet (1946). The final method by which/

which these cells were prepared, and the steps for setting up the titrations have been described previously in the chapter referred above.

As a preliminary titration, paired sera from four patients were examined with the following results:

Patient	Days after onset	Reciprocal of final serum dilution				
		20	40	100	200	400
C.HU.	3	0	0	0	0	0
	17	4	4	±	0	0
P.SW.	9	0	0	0	0	0
	32	0	0	0	0	0
R.SW.	2	4	4	0	0	0
	15	4	4	4	±	0
I.MAC	14	0	0	0	0	0
	63	0	0	0	0	0

0: No agglutination

4: Strong agglutination

The titrations showed that with two of the paired sera there was no agglutination even in convalescent samples. A different batch of sensitised cells were prepared with James strain of virus from a subsequent allantoic passage. Paired sera from 15 patients, including the sera from three of the patients/

patients (P.SW., R.SW., and I.Mac.) titrated previously, were tested. Although the sensitised cells were found to conform to the 'receptor gradient' and did not auto-agglutinate, none of the convalescent sera showed any rise of the agglutinating antibody. Even the sera from R.SW. which previously demonstrated the antibody, failed to show any positive reaction. It is not known whether the effect was due to the low titre of the "cell sensitising agent" as described by Lind (1948). No attempt was made to determine this sensitising index. Further titrations of the sera were not made by the Burnet test.

Comparisons of the antibody levels in acute and convalescent specimens obtained by different methods of titration.

The titres of individual or paired sera from mumps patients as obtained by the haemagglutination inhibition, neutralisation and complement fixation tests have been already described. The comparison is therefore limited to the discussion of the results which will help in elucidating the diagnostic usefulness of the different tests.

It was apparent from these titrations that each of/

of the above tests has its merits and drawbacks, and may be useful for the laboratory diagnosis of mumps. Considerable difficulty was experienced in standardising the Burnet test and because of the irregular results it was not used for comparative titrations.

In paired samples of sera where the initial specimen was obtained before eight days a significant increase in the amount of antibody could be demonstrated in practically all the cases by the H.I., neutralisation tests and by the complement fixation test using viral antigen (table ..V..). Due to individual variations, in some cases the initial sample of serum may show an earlier appearance of the antibody, so that a fold-increase in titre is not demonstrable. Such variation was less marked with the H.I. and neutralising antibodies than with the complement fixing antibodies. Of the latter, the anti-viral antibody (Anti-V) could be demonstrated in all the convalescent sera, although in isolated cases it appeared to develop late. Wider variation in the titres of the Anti-S has been found to occur (fig. 1..,c). In two cases it failed to develop even in the convalescent sera.

Of the four antibodies (H.I., neutralising, Anti-V/

Anti-V and Anti-S), it became apparent that the Anti-S was the earliest to attain a significant level. It could be detected in majority of the sera within five to six days after onset. A high level may be attained even within 2-3 days. The neutralising and H.I. antibodies are detected within 7-8 days and it appears that the former increases rapidly of the two. During the early second week of the disease (9-10 days) the Anti-S attains maximum level and significant increase in titre (4 to 8 fold) of the neutralising and haemagglutination inhibiting antibodies is always demonstrated. The Anti-V is found at significant level in majority of the samples but individual serum may still be negative.

The Anti-S, neutralising, H.I. and Anti-V antibodies attain their maximum titres in that order during the course of the illness. Numerically higher titres are obtained with the neutralising and H.I. antibodies than the Anti-V. But in paired samples the Anti-V may show high fold-increase in titre similar to the neutralising antibody. The Anti-S never attain a high titre and as explained earlier may not show a comparable rise in titre unless the initial sample is collected within 1-2 days.

Just/

Just as their rise differs so these antibodies are not maintained at the same constant level for any length of time. The Anti-S declines more rapidly than the other antibodies, and is reduced to a minimum after three months. The neutralising antibody also shows a fall similar to the Anti-S but probably starts later and some samples may still show moderate content of the antibody after three months. The Anti-V usually shows significant and high titres even at the end of three months and in most of the samples collected after that period the antibody is present well above the minimum significant level. The fall in the level of the H.I. antibody is least marked and a high titre, in comparison to that of the acute stage samples, is maintained long after the disease has subsided (figs. 1.(a) & 3).

The antibody levels in four different mumps patients are shown in fig...5... Four or more samples of sera were obtained from each patient. From the saliva of one of the patients (P.SW.) the mumps virus was isolated on the 1st. day of the disease. In patients I.Ma. and J.St. the initial blood samples were obtained after ten days. The graphs in general conforms to what has been described above, but individual variations may also be observed.

Titration/

Titration of haemagglutination - inhibiting antibody against different strains of the mumps virus.

Several acute and convalescent samples of sera from mumps patients were titrated against three different strains of the mumps virus (Enders, James and Paul). The experiment was undertaken to find out whether the use of different strains of virus would give identical titre of the H.I. antibody.

Eighteen acute sera (eight days or before) and 19 convalescent (after eight days) sera were titrated. Of these, 30 paired sera were obtained from 15 patients. The virus dilutions were standardised to contain eight units per ml. The sera were diluted in bulk and each dilution was distributed into three series. The three strains of the virus were added in these serial dilutions separately. The tests were completed as described before.

The results are shown in table .VII.... The titres of the paired sera and two convalescent sera are shown in table ..VIII.. An analysis of the results show that the titre of each serum is not always the same when different strains of virus are used. The results may be summarised as below:

No./

	Total	Acute Sera	Con-valesc. Sera
No. of sera with same end point against all the three virus strains ...	11	7	4
No. of sera with same end point against two strains	21	9	12
No. of sera with different end points	5	2	3
Total:	37	18	19

It will be observed that only in 11 sera out of 37 the titres were identical against different strains. In 21 sera at least one of the strains did not give the same end point and there was a 2-fold difference in the titre. The difference was more marked with the convalescent sera. Although it may be argued that a 2-fold difference in titre is not significant, yet under the conditions of the test it is probable that some of the sera protrayed a true difference of the end-point (see Chapter. III. on the standardisation of the H.I. test). However, at least in 5 sera there was a four-fold difference between the minimum and the maximum titres (marked with asterisks in tables . VII. and VIII.), as obtained against the three strains.

If/

If the fold-rise in titres of the paired sera is considered, it will be found that, in five patients the fold-rise was the same against all the three strains. In another five patients the fold-rise was identical with only two strains and different with the third one. The rest of the five patients showed different fold-rise with different strains of the virus, as shown in this table.

Patient	Fold rise		
	Enders Strain	James Strain	Paul Strain
C.DO.	8	4	16
M.YU.	8	16	64
B.LO.	32	16	8
A.CU.	32	64	16
N.ST.	0	4	4

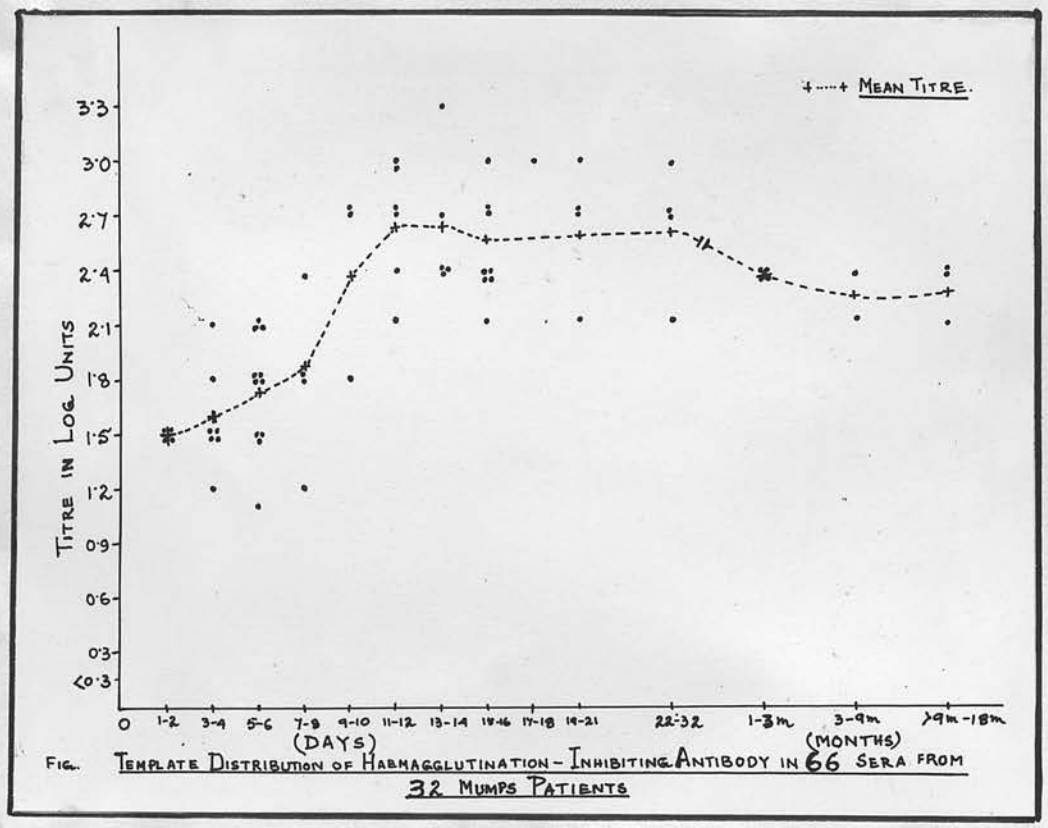
The above analysis shows that in at least six patients out of 15 the use of different virus strains may reveal significant differences either in the fold-rise or in the titre of individual serum (J.Dr. Sample 2). The findings in the sera of patients M.YU. and N.ST. are also of some interest. In both cases the convalescent serum gives much higher titre when titrated against the James and the Paul strains. Besides, it will be seen/

seen that with the Enders strain there is no difference in the titres of the paired sera from N.St.; on the other hand, against James and Paul strains there is evidence of further rise of the antibody in the convalescent sample. The following table tends to show that both the James and the Paul strains will give higher end points when used for the H.I. test. Of the two, the James strain indicates slightly higher levels of the acute sera. The Paul strain shows antibody levels of acute sera comparable to those obtained against the Enders strain; with the convalescent sera it definitely indicates higher level than that against the Enders strain.

Table ...IX....

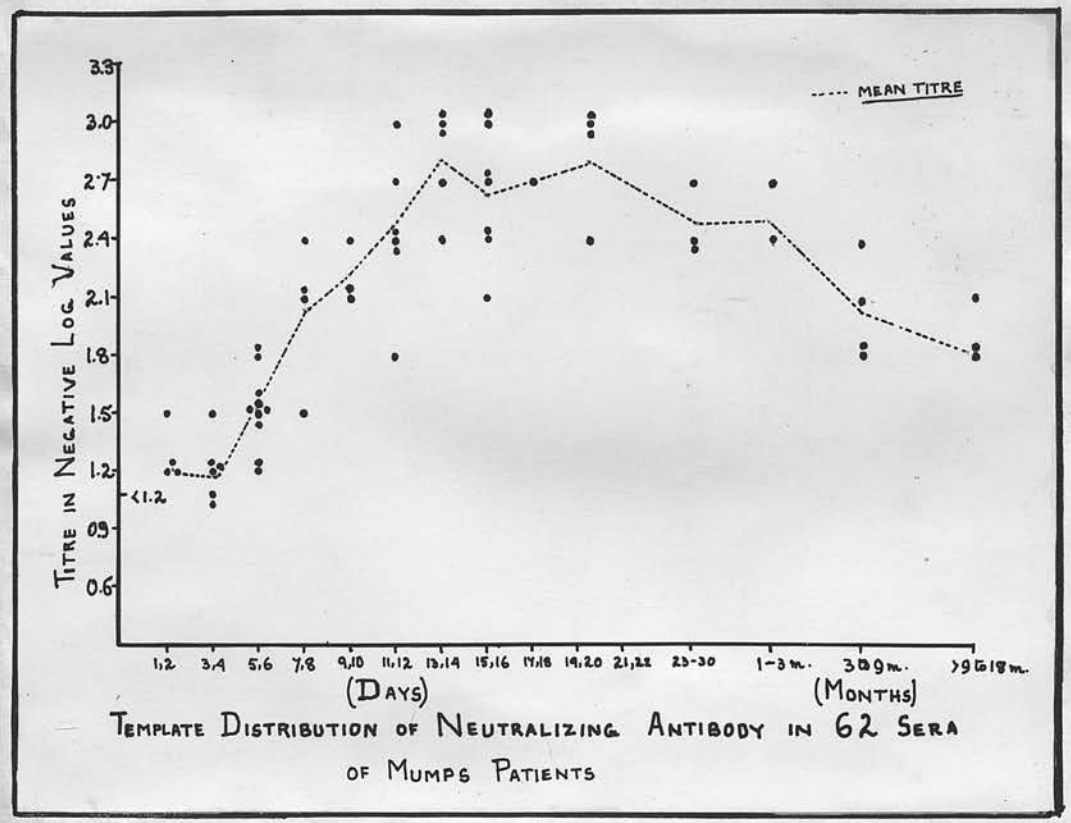
Correlation of the number of sera with a particular titre of the H.I. antibody and the strain of mumps virus used for the titration.

Sample	Virus	Reciprocal of the titres							
		16	32	64	128	256	512	1024	2048
Acute (Total = 18)	Enders	1	8	6	2	1			
	James		4	10	4				
	Paul	2	6	7	2	1			
Convalesc (Total = 19)	Enders				3	7	4	4	1
	James				1	5	4	5	4
	Paul			1	1	3	5	9	



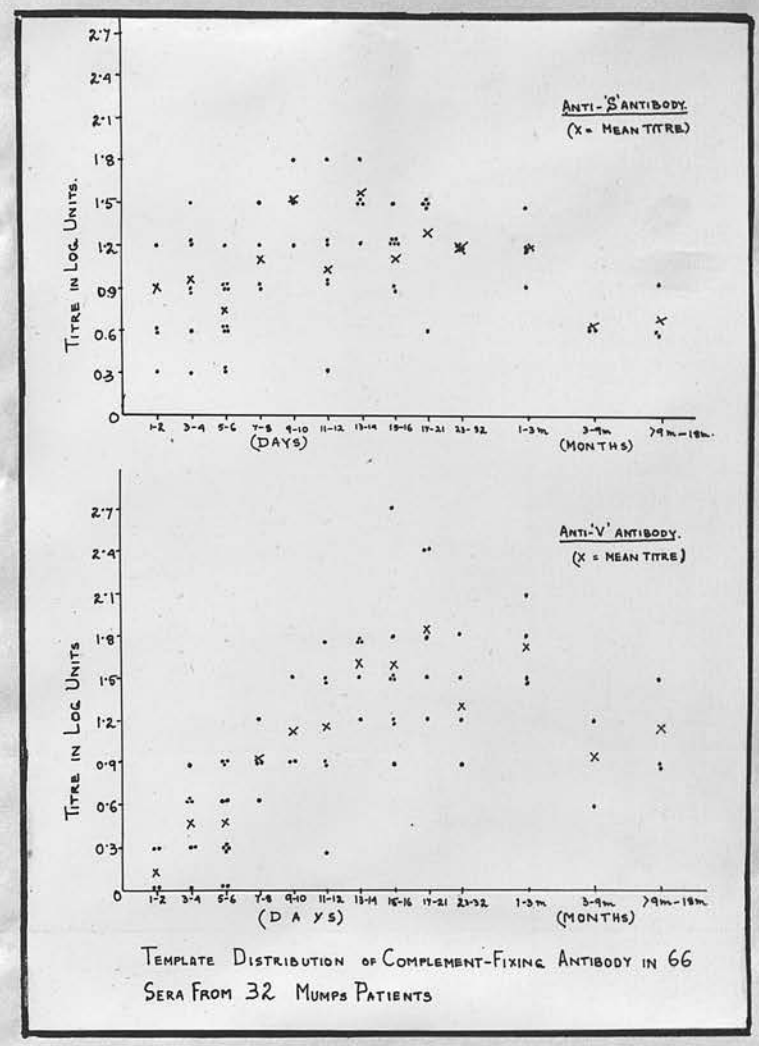
(a)

FIG. TEMPLATE DISTRIBUTION OF HAEMAGGLUTINATION-INHIBITING ANTIBODY IN 66 SERA FROM 32 MUMPS PATIENTS



(b)

TEMPLATE DISTRIBUTION OF NEUTRALIZING ANTIBODY IN 62 SERA OF MUMPS PATIENTS



(c)

(d)

TEMPLATE DISTRIBUTION OF COMPLEMENT-FIXING ANTIBODY IN 66 SERA FROM 32 MUMPS PATIENTS

Fig. 1. a, b, c, and d. Template distribution of antibody levels in mumps sera as obtained by different tests. Individual variations on any particular day after the onset of the disease are also seen.

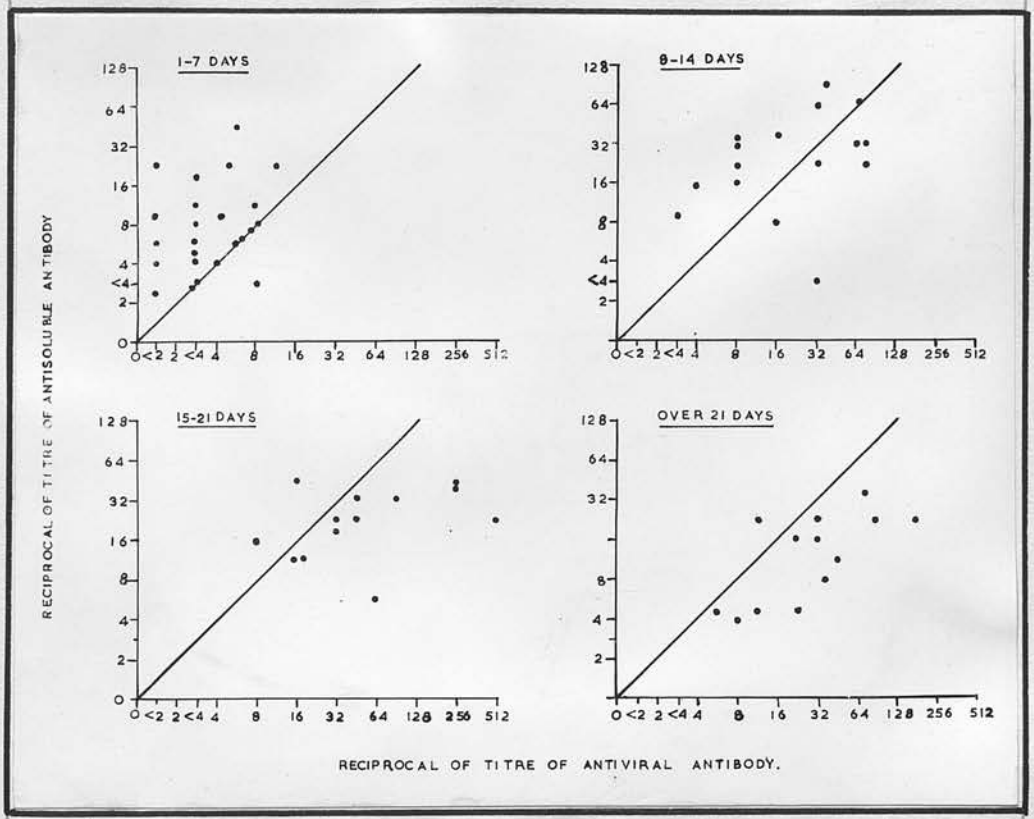


Fig. 4.

Relation of anti-S and anti-V (complement-fixing antibody) responses in 66 sera from mumps patients.

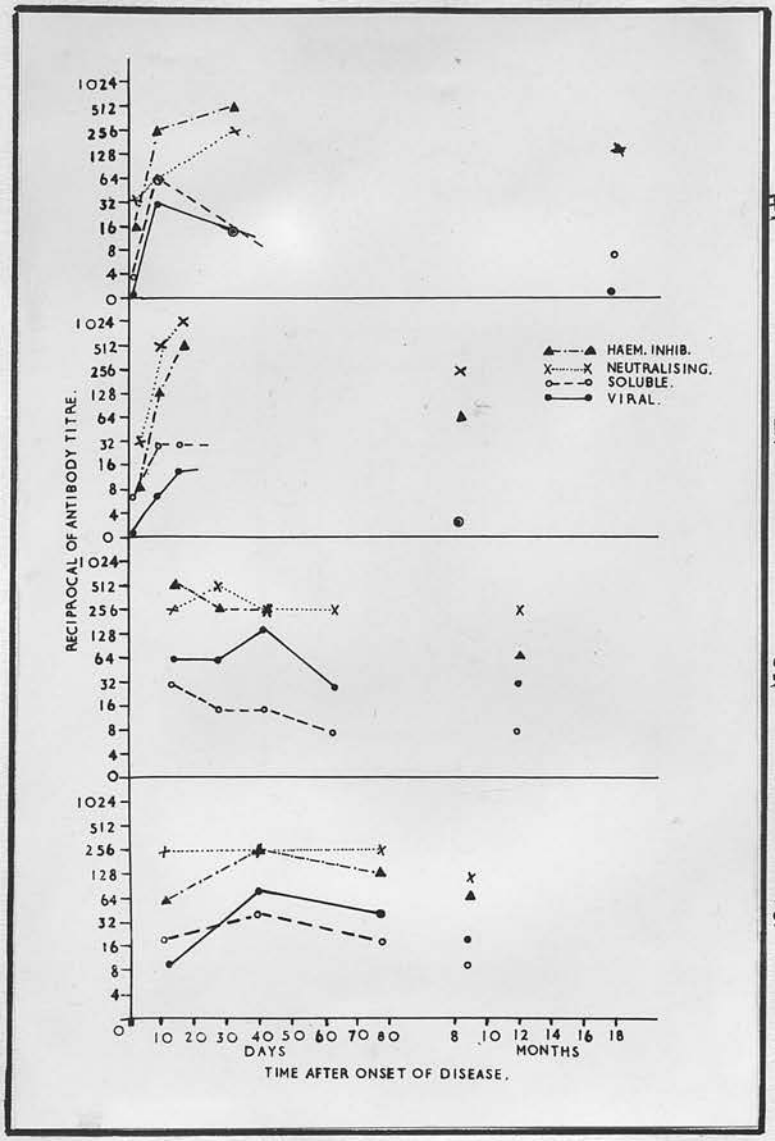


Fig. 5.

Antibody levels in four mumps patients where several blood samples could be obtained over a prolonged period. Letters on right stand for their initials.

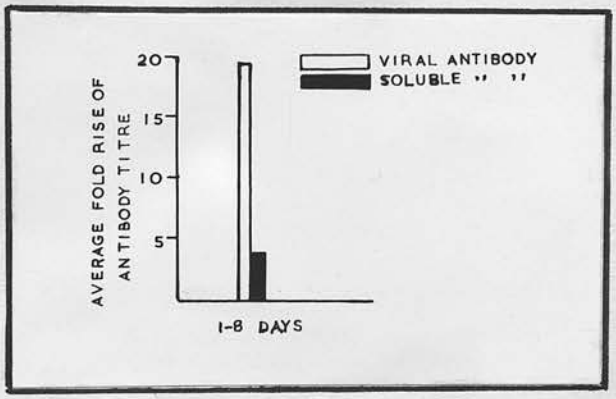


Fig. 6 to show that the average fold-rise of the "viral"antibody is very high (about 5 times more) in comparison to that of the "soluble" antibody, especially when the first sample of serum is obtained in acute stage of the disease and the subsequent sample after an interval of at least 8 days.

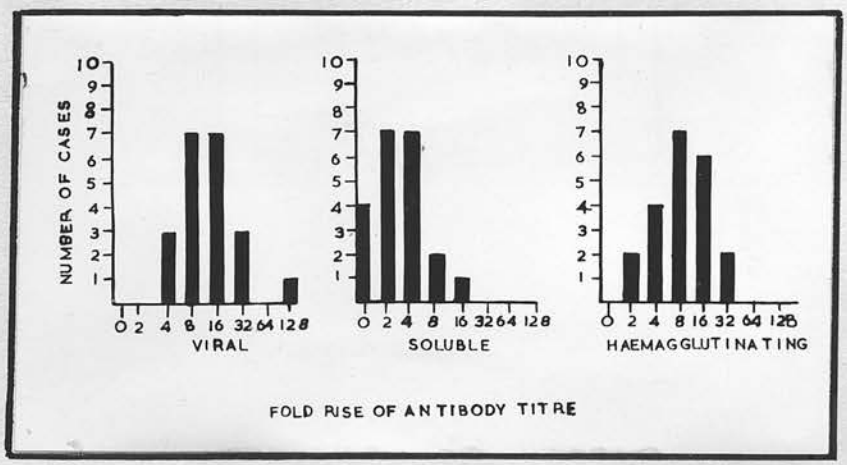


Fig. 7. Fold-rise of antibody titre between first and second samples of sera obtained from 21 mumps patients. In these cases the initial samples were all collected within 8 days after onset of the disease.

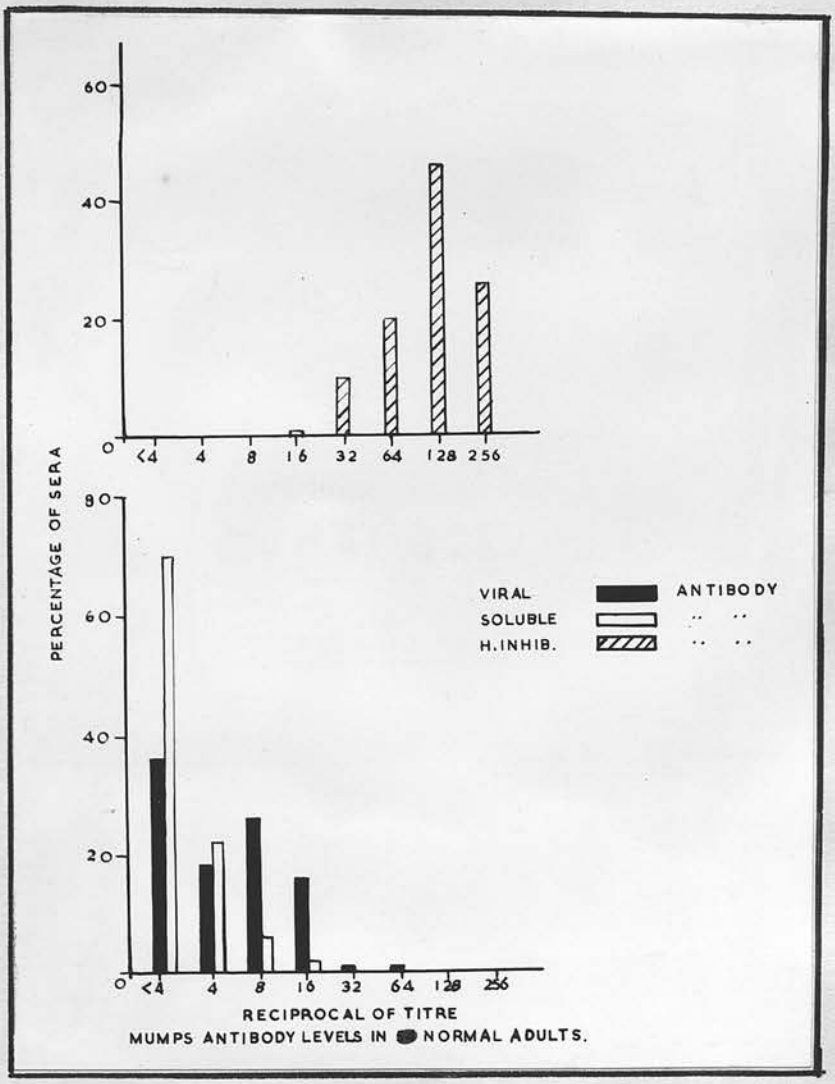

60
40
20
0
<4
4
8
16
32
64
128
256
80
60
40
20
0
PERCENTAGE OF SERA
VIRAL ANTIBODY
SOLUBLE .. ..
H.INHIB. .. ..
RECIPROCAL OF TITRE
MUMPS ANTIBODY LEVELS IN NORMAL ADULTS.

Fig. 8.

TABLE: I

ANTIBODY LEVEL IN THE SERA OF 31 MUMPS PATIENTS AGAINST MUMPS AND NEWCASTLE DISEASE VIRUSES.

No.	Patient	Sample No.	Days after onset	Rise of antibody against mumps virus (ENDERS Strain).								Rise of antibody against N.D.V. (LONDON Strain)			
				H.I.antibody		Complement - fixing. Against "Viral" antigen.		Complement - fixing. Against "Soluble" antigen.		Neutralising.		Haemagglutination inhibiting.		Complement - fixing. Against infected allantoic fluid components.	
				Titre	Maximum rise (-fold)	Titre	Maximum rise (-fold)	Titre	Maximum rise (fold)	Titre	Maximum rise (-fold)	Titre	Maximum rise (fold	Titre	Maximum rise (fold)
1	P.Sw.	I. II, III. IV.	1 9 32 18 (months)	32(1) 64(2) 256(2) 128(1)	X8	<2 32(1) 16(0) 8(2)	X32	4(2) 64(0) 16(2) 4(2)	X16	16 256 512 128	X32	<32 256(1) N.D. N.D.	X16	<4 <4 8(1) <4	X4
2	R.Sw.	I. II. III.	2 15 18 (months)	32(1) 256(0) 256(2)	X8	<4 32(2) 8(0)	X16	4(0) 16(1) 4(1)	X4	16 256 64	X16	32(2) 128(2) 128(2)	X4	<4 8(2) N.D.	X4
3	I.Mac.	I. II. III. IV. V.	14 27 42 63 12 (months)	256(1) 512(2) 256(0) 256(1) 256(1)	X2	64(2) 64(0) 128(2) 32(0) 32(1)	X2	32(2) 16(0) 16(0) 8(0) 8(2)	X0	512 256 256 N.D. 64	X0	64(0) N.D. 64(0) N.D. 128(2)	X2	4(2) N.D. 8(0) N.D. N.D.	X2
4	C.Hu.	I. II. III. IV.	3 10 17 8½ (months)	32(1) 512 (2) 1024(2) 256(0)	X32	<2 8(2) 16(2) 4(0)	X16	8(1) 32 (2) 32(0) 4(1)	X4	<16 128 512 64	X64	N.D. 128(0) 256(0) N.D.	X2	<4 4(2) 16(2) 4(2)	X8
5	J.St.	I. II. III. IV.	11 40 78 9 (months)	256(2) 256(0) 256(1) 128(2)	X0	8(2) 64(1) 32(2) 16(0)	X8	16(0) 32(1) 16(0) 8(1)	X2	64 256 128 64	X4	128(0) 128(2) N.D. N.D.	X0	4(2) <4 <4 <4	X0
6.	I.Met.	I. II. III.	8 19 26	256(1) 512(2) 512(2)	X2	8(2) 32(0) 8(0)	X4	32(2) 32(2) 16(0)	X0	256 1024 512	X4	64(2) 128(2) 32(0)	X2	4(2) 8(0) 8(0)	X2
7.	S.Woo.	I. II.	11 16	128(2) 1024(0)	X8	<4 32(2)	X16	8(1) 16(0)	X2	256 512	X2	32(1) 256(2)	X8	4(0) 8(2)	X2
8.	D.Lo.	I. II.	5 15	64(1) 512(2)	X8	4(0) 16(2)	X4	4(0) 8(0)	X2	N.D. N.D.	—	32(2) 64(2)	X2	<4 <4	X0
9.	S.E.M.	I. II.	3 14	16(0) 256(0)	X16	4(1) 64(2)	X16	16(0) 64(1)	X4	16 1024	X64	N.D. 64(1)	X ? 2	<4 <4	X0

(Explanatory note at the end of the Table).

ANTIBODY LEVEL IN THE SERA OF 31 MUMPS PATIENTS AGAINST MUMPS AND NEWCASTLE DISEASE VIRUSES (Contd.)

No.	Patient	Sample No.	Days after onset.	Rise of antibody against mumps virus (ENDERS Strain).						Rise of antibody against N.D.V. (LONDON Strain)			
				H.I.Antibody		Complement - fixing Against "Viral" antigen.		Complement - fixing Against "Soluble" antigen.		Neutralising.		Haemagglutination inhibiting.		Complement - fixing Against infected allantoic fluid components	
				Titre	Maximum rise (-fold)	Titre	Maximum rise (-fold)	Titre	Maximum rise (fold)	Titre	Maximum rise (-fold)	Titre	Maximum rise (fold)	Titre	Maximum rise (fold)
10	J.Wh.	I. II.	3 12	32(0) 512(1)	X16	4(0) 32(2)	X8	32(0) 64(2)	X2	16 1024	X64	N.D. 512(0)	X ? 16	<4 8(1)	X4
11	Y.Ma.	I. II.	2 16	32(1) 256(0)	X8	<2 32(0)	X32	16(0) 16(0)	X0	32 256	X8	<32 128(0)	X8	<4 8(0)	X4
12	M.Ir.	I. II.	5 13	<16 256(2)	X32	<4 32(2)	X16	4(0) 16(0)	X4	32 1024	X32	<16 <16	X0	<4 <4	X0
13	I.Ba.	I. II.	3 11	32(2) 512(0)	X16	<4 32(2)	X16	<4 <4	X0	16 512	X32	<16 <16	X0	<4 <4	X0
14	A.Cu.	I. II.	5 13	64(2) 2048(0)	X32	<4 64(1)	X32	8(2) 32(2)	X4	32 1024	X8	<16 512(0)	X64	<4 16(2)	X8
15	B.Loc.	I. II.	7 20	16(1) 512(0)	X32	8(2) 256(2)	X32	8(2) 32(0)	X4	128 1024	X8	<16 32(2)	X4	<4 <4	X0
16	J.Dr.	I. II.	5 20	32(2) 128(0)	X4	<4 256(2)	X128	4(0) 32(0)	X8	32 1024	X32	16(2) 32(0)	X2	<4 4(1)	X2
17	J.Bo.	I. II.	2 15	32(2) 128(2)	X4	<4 16(1)	X8	<4 8(0)	X4	<16 1024	X128	<16 <16	X0	<4 <4	X0
18	J. Dou.	I. II.	6 19	128(0) 1024(0)	X8	8(2) 64(2)	X8	<4 4(0)	X2	32 256	X8	<16 128(2)	X16	<4 4(1)	X2
19	B.Ca.	I. II.	6 23	128(0) 1024(0)	X8	<4 32(2)	X16	8(0) 16(2)	X2	16 256	X16	<16 <16	X0	<4 <4	X0
20	I.Mic.	1. II.	4 12	128(1) 1024(0)	X8	8(2) 64(2)	X8	8(2) 8(0)	X0	32 256	X8	16(1) 128(0)	X8	<4 16(2)	X8
21	W.Gi.	I. II.	5 11	64(1) 1024(0)	X16	8(2) 64(1)	X8	8(0) 16(0)	X2	32 256	X8	<16 256(2)	X32	<4 8(2)	X4

(Explanatory note at the end of the Table).

ANTIBODY LEVEL IN THE SERA OF 31 MUMPS PATIENTS AGAINST MUMPS AND NEWCASTLE DISEASE VIRUSES (Contd.)

No.	Patients	Sample No.	Days after onset	Rise of antibody against mumps virus (ENDERS Strain).								Rise of antibody against N.D.V. (LONDON Strain)			
				H.I.antibody		Complement - fixing Against "Viral" antigen		Complement - fixing Against "Soluble" antigen		Neutralising.		Haemagglutination inhibiting.		Complement - fixing Against infected allantoic fluid	
				Titre	Maximum rise (-fold)	Titre	Maximum rise (-fold)	Titre	Maximum rise (fold)	Titre	Maximum rise (-fold)	Titre	Maximum rise (fold)	Titre	Maximum rise (fold)
22	J.Ja.	I. II.	8 15	64(1) 256(0)	X4	16(2) 512(2)	X32	8(2) 16(0)	X2	128 1024	X8	<16 32(0)	X4	<4 <4	X0
23	C.Dor.	I. II.	5 16	32(2) 256(1)	X8	<2 8(2)	X8	2(1) 16(2)	X8	64 512	X8	16(2) 128(1)	X8	<4 8(0)	X4
24	G.Be.	I. II.	5 16	128(2) 512(1)	X4	8(0) 64(0)	X8	16(0) 32(2)	X2	64 1024	X16	32(2) 128(0)	X4	<4 16(1)	X8
25	N.Br.	I.	13	512(0)	-	16(2)	-	32(1)	-	256	-	32(2)	-	4(0)	-
26	C.Coo.	I.	9	512(1)	-	8(2)	-	16(2)	-	128	-	64(0)	-	<4	-
27	H. La.	I.	4	64(1)	-	2(0)	-	16(1)	-	<16	-	32(2)	-	<4	-
28	G.Fa.	I.	8	64(2)	-	4(2)	-	16(2)	-	32	-	<32	-	<4	-
29	J.McN.	I.	5	64(2)	-	4(1)	-	8(1)	-	16	-	<32	-	<4	-
30	S.Mc.	I.	6	32(1)	-	<2	-	4(0)	-	32	-	<32	-	<4	-
31	N.Ro.	I.	3	32(0)	-	4(0)	-	4(0)	-	N.D.	-	<32	-	<4	-

N.B. The titres for any test represent the reciprocal of the dilution of the serum; the figures in brackets denote the degree of reaction as explained in the text.
N.D. denotes that the examination has not been made with the serum for presence of the particular antibody content.

TABLE : II

The Antibody Level in Human Sera After Mumps Infection.

Time after onset of the disease.	Number of Sera.	Titre of Haemagglutination - inhibiting antibody	Titre of the complement fixing antibody		Titre of the neutralising antibody
			Against viral antigen	Against soluble antigen.	
1 day	1	32(1),	<2,	4(2),	16
2 days	3	32(1), 32(1), 32(2),	<4, <2, <4,	4(o), 16(o), <4,	16, 32, <16,
3 "	5	16(o), 32(o), 32(o), 32(2), 32(1),	4(1), 4(o), 4(o), <4, <2	16(o), 4(o), 32(o), <4, 8(1),	16, - 16, 16, <16,
4 "	2	64(1), 128(1),	2(0), 8(2)	16(1), 8(2),	<16, 32,
5 "	8	<16, 32(2), 32(2), 64(1), 64(1), 64(2), 64(2), 128(2),	<4, <4 <2 8(2), 4(o), 4(1). <4, 8(o)	4(2), 4(o), 2(1), 8(o), 4(o), 8(1), 8(2), 16(o),	32, 32, 64, 32, - 16, 32, 64,
6 "	3	32(1), 128(o), 128(2),	<2, 8(2), <4,	4(o), <4, 8(o),	32, 32, 16
7 "	1	16(1),	8(2),	8(2),	128,
8 "	3	64(1), 64(2), 256(1),	16(2) 4(2), 8(2)	8(2) 16(2) 32(2)	128, 32, 256
9 "	2	64(2), 512(1),	32(1), 8(2),	64(0), 16(2)	256, 128,
10 "	1	512(2),	8(2),	32(2)	128,
11 "	4	256(2), 512(0), 1024(0) 128(2),	8(2), 32(2), 64(1) <4	16(0), <4, 16(0), 8(1)	64, 512, 256, 256,
12 "	2	512(1), 1024(0),	32(2) 8(2),	64(2), 8(2),	1024, 256,
13 "	3	256(2), 512(2), 2048(0),	32(2), 16(2), 64(1)	16(0) 32(1) 32(1),	1024, 256, 1024,
14 "	2	256(0), 256(1),	64(2), 64(2),	64(1), 32(2),	1024, 512,
15 "	4	128(2), 256(0), 256(0), 512(2),	16(1) 512(2), 32(2), 16(2)	8(0), 16(0), 16(1), 8(0)	128, 1024, 256 -,
16 "	4	256(0), 256(1), 512(2), 1024(0),	32(0), 8(2), 64(0), 32(2),	16(0), 16(2), 32(2), 16(0),	256, 512, 1024, 512,
17 "	1	1024(1)	16(2)	32(0),	512,
19 "	2	512(2), 1024(0),	32(0), 64(2),	32(2) 4(0)	1024, 256,
20 "	2	128(0), 512(2),	256(2), 256(2),	32(0), 32(0),	1024, 1024,

(Explanatory notes at the end of the Table).

TABLE : II (contd.).

Time after onset of the disease.	Number of Sera.	Titre of Haemagglutination - inhibiting antibody *	Titre of the complement fixing antibody.		Titre of the neutralising antibody.
			Against viral antigen	Against soluble Antigen.	
23 Days	1	1024(2)	32(2)	16(2)	256
26 "	1	512(1)	8(0)	16(0)	512
27 "	1	512(2)	64(0)	16(0)	256,
32 "	1	128(2)	16(0)	16(2)	512,
40 "	1	256(2)	64(1)	32(1),	256,
42 "	1	256(0)	128(0)	16(0),	-
63 "	1	256(1),	32(0),	8(0),	256,
78 "	1	256(1),	32(2),	16(0),	128,
8½ months	1	256(0),	4(0),	4(1),	64,
9 "	1	128(2)	16(0),	4(1),	64,
12 "	1	256(1)	32(1),	8(2)	64,
18 "	2	128(2),	8(2), 8(0),	4(2), 4(1),	128, 64,

N.B. 1). The titres of sera are expressed as the reciprocal of initial dilution for the complement fixing antibodies and that of the final dilution for the haemagglutination - inhibiting and neutralising antibodies.

2). The figures in brackets express the degree of reactions for any particular test, as explained in the text.

3). Neutralising antibodies are expressed as N.T.50.

4). The values in each column have been so arranged that they represent the antibody levels for any particular serum obtained by the appropriate test..

TABLE : III

The Antibody Level in Human Sera After Mumps Infection (Calculated mean values).

Time after onset of the Disease.	Haemagglutination inhibiting antibody (mean titre)		Complement fixing antibody titre (mean titre).				Neutralising antibody (Mean Titre)	
			Against "Viral" Antigen.		Against "Soluble" Antigen.			
	Value in Negative log.	Reciprocal of the geometric mean.	Value in negative log.	Reciprocal of the geometric mean.	Value in negative log.	Reciprocal of the geometric mean.	Value in negative log.	Reciprocal of the geometric mean.
1 to 2 days	1·5	32	0·15	1·4	0·9	8	1·2	16
3 to 4 "	1·6	39	0·47	2·95	0·94	8·7	1·15	14
5 to 6 "	1·75	56	0·46	2·8	0·71	5.1	1·5	32
7 to 8 "	1·8	63	0·9	8	1·12	13	2·0	100
9 to 10 "	2·4	251	1·1	12·6	1·5	32	2·2	159
11 to 12"	2·65	447	1.15	14	1·05	11	2.45	282
13 to14 "	2.64	437	1·62	42	1·5	32	2·83	676
15 to16 "	2.5	316	1·54	35	1·16	14.5	2·62	417
17 to 21 "	2·7	500	1·86	72	1·32	21	2·83	676
22 to 32 "	2·6	400	1·3	20	1·2	16	2·5	316
>1 to 3 Months.	2·4	251	1·72	52	1·2	16	2.55	360
3 to 9 Months.	2·25	178	0·9	8	0·6	4	2·0	100
>9 to 18 Months.	2·3	200	1·1	12.6	0·7	5	1·9	80

TABLE : IV

Titre of the Neutralising Antibody in Sera of 29 Mumps Patients

Name	Days after onset	*Titre
N.Hu.	3	<16
	10	128
	17	512
	8 1/2 (months)	64
R.Sw.	2	16
	15	256
	1 1/2 (years)	64
J.Do.	6	32
	19	256
B.Ca.	6	16
	23	256
I.Ma.	14	512
	27	256
	63	256
	1 (year)	64
P.Sw.	1	16
	9	256
	32	512
	1 1/2 (years)	128
C.Do.	5	64
	16	512
G.Be.	5	64
	16	1024
Y.Ma.	2	32
	16	256
J.St.	11	64
	40	256
	78	128
	9 (months)	64
S.Wo.	11	256
	16	512
J.Wh.	3	16
	12	1024
J.Ba.	3	16
	11	512
S.Ell.	3	16
	14	1024
W.Gi.	5	32
	11	256
A.Cu.	5	32
	13	1024
M.Ir.	5	32
	13	1024
I.Mi.	4	32
	12	256
J.Ja.	8	128
	15	1024
J.Bo.	2	<16
	15	1024
B.Lo.	7	128
	20	1024
J.Dr.	5	32
	20	1024
I.Me.	8	256
	19	1024
	26	512
N.Br.	13	256
H.La.	4	<16
J.Mc.	5	16
S.Mc.	5	32
G.Fa.	8	32
C.Co.	9	128

*Reciprocal of the final serum dilution

TABLE : V

Fold-rise in Titre ofMumps Antibodies in 21 Patients Where the Initial Sample of Serum Was Collected On Or Before 8 Days After Onset

Fold-rise	Antibody			
	H.I.	Anti-V (c.fix.)	Anti-S (c.fix.)	Neutr-alising
0			4^^	
2	1+		7	
4	4	3	7	1
8	8	7	2	8
16	4	7	1	3
32	4	3		4
64				3
128		1		1

^^= number of sera.
+ = the initial sample of sera was collected on 8 th. day and had a titre of 1:256 ; a maximum titre of 1:512 was seen in the samples of 19th. and 26th.days.

TABLE : VI

Fold-rise in Titre of Mumps Antibodies in Three Patients Where the Initial Sample Of Serum Was Collected After 8 Days

Patient	1st.sample collec. on	Fold-rise			
		H.I.	Anti-V	Anti-S	Neutral.
S.Woo.	11	x8	16	2	2
J.St.	11	0	8	2	4
L.Mac.	14	2	2	0	0

x :fold-rise

TABLE : VII

HAEMAGGLUTINATION - INHIBITION TITRE OF ACUTE AND CONVALESCENT HUMAN SERA WITH THREE DIFFERENT STRAINS OF MUMPS VIRUS.

(A) Acute Samples (8 days or less).

Enders -strain	James -strain.	Paul -strain.
64	64	64
64	64	64
32	64	64
128	128	128
32	64	32
256	128	256
128	128	128
32	64	64
32	64	16*
32	64	32
16	32	32
64	32	64
32	64	32
64	64	64
64	128	32*
64	64	64
32	32	32
32	32	16

(B) Convalescent Samples (After 8 days).

Enders -strain	James -strain	Paul -strain.
1024	1024	1024
256	512	512
128	256	512*
1024	1024	1024
256	256	256
512	256	512
512	1024	1024
256	256	1024*
256	1024	1024*
1024	2048	1024
128	128	64
1024	2048	1024
512	512	256
2048	2048	1024
256	1024	512*
128	256	128
256	512	256
256	2048	1024*
512	512	512

N.B. The figure denotes the reciprocal of the final dilution of the serum.

* See text

TABLE: VIII

HAEMAGGLUTINATION - INHIBITION TEST OF HUMAN SERA AGAINST THREE DIFFERENT STRAINS OF THE MUMPS VIRUS.

Patient	Sample.	Days after onset.	H. Inhibition Titre ¥		
			Against Enders Strain.	Against James Strain.	Against Paul Strain.
W.Gi.	1 2	5 11	64(1) 1024(0)	64(0) 1024(0)	64(2) 1024(2)
J.Ja.	1 2	8 15	64(1) 256(0)	64(0) 512(0)	64(2) 512(2)
J.Dr.	1 2*	5 20	32(2) 128(0)	64(2) 256(1)	64(2) 512(2)
J.Mi.	1 2	4 12	128(1) 1024(0)	128(0) 1024(2)	128(2) 1024(2)
R.Sw.	1 2	2 15	32(1) 256(0)	64(2) 256(1)	32(0) 256(0)
I.Mc.	1 2	8 19	256(1) 512(2)	128(2) 256(0)	256(2) 512(2)
G.Be.	1 2	5 16	128(2) 1024(2)	128(0) 1024(0)	128(0) 1024(2)
C.Do.	1 2*	5 16	32(2) 256(1)	64(1) 256(0)	64(2) 1024(2)
M.Yu.	1 2*	2 16	32(1) 256(0)	64(1) 1024(0)	16(0) 1024(1)
N.Hu.	1 3	3 17	32(1) 1024(2)	64(2) 2048(0)	32(0) 1024(0)
S.Wo.	1 2	11 16	128(2) 1024(0)	128(0) 2048(2)	64(0) 1024(0)
B.Lo.	1 2	7 20	16(1) 512(2)	32(0) 512(2)	32(2) 256(1)
A.Cu.	1 2	5 13	64(2) 2048(0)	32(0) 2048(0)	64(2) 1024(0)
J.Bo.	1 2	2 15	32(2) 128(2)	64(1) 256(1)	32(2) 128(2)
N.St.	1 2*	11 40	256(2) 256(0)	512(2) 2048(1)	256(0) 1024(2)
C.Co.	1	9	512(1)	512(0)	512(0)
I.Ma.	1*	14	256(1)	1024(0)	512(1)

¥ The titre expressed as the reciprocal of the final dilution of the serum; the figures in brackets indicate the degree of inhibition from the pattern of haemagglutination reactions.

* see text

TABLE : X

The Antibody Levels in 26 Mumps Sera Collected Within 8 Days After the Onset of the Disease

Titre (reciprocal)	Number of sera			
	H.I. test	C.F. test		Neutr. test
		Anti-V	Anti-S	
<4	-	13	4	-
4	-	6	7	-
8	-	6	8	-
16	3+	1	5	10+
32	11		2	9
64	7			2
128	4			2
256	1**			1**
Total	26	26	26	24

\+ includes samples with titre of less than 1:16

** serum from a case on 8th.day.

DISCUSSION

The results of the present study show a rise of antibody in all typical cases of mumps parotitis. The antibody against mumps virus may also be demonstrated in patients with unusual manifestations, such as, the involvement of the nervous system or the submanibular and sublingual salivary glands.

Some of the data presented here point to the multiple nature of the mumps antibody. The immune antibody components which are recognisable by different tests mentioned above have different characteristics, e.g.

(i) their time of appearance in the sera is at different periods of the disease;

(ii) they increase in amounts independently;

(iii) they persist at different levels during and after the illness;

(iv) one or other of the antibodies may even fail to develop, and

(v) their individual levels cannot be correlated with the severity of the infection. It is possible that each antibody response corresponds to the antigenic configuration of the mumps/

mumps virus and the variation in response is modified according to the strain of the virus responsible for the infection. However the development of these antibodies appears to conform to some definite pattern. A study of their nature has therefore proved helpful in the proper assessment of the serological diagnosis in mumps.

The titration of the antibody levels by different tests has shown their usefulness for diagnostic purposes. The specificity of the H.I. and C.F. tests has been well proved by earlier investigators. The neutralisation test 'in ovo' which has not been applied hitherto to a study of this nature appears to be promising. Its reliability as a diagnostic aid is shown by the fact that in every case of typical mumps as well as in the atypical cases, significant and high fold-increase in titre has been demonstrated in paired sera.

In connection with the neutralisation test it must be admitted that the antibody titrations were not carried out in accordance with the standard practice of using large number of eggs for each dilution of the serum-virus mixture. Yet, with the smaller number of eggs for each dilution definite increase or decrease of antibody level could be observed in serial samples of sera. By the use

of/

of smaller amount of virus as test antigen, a greater range of titre between the acute and convalescent sera has been achieved. The wider range, it is felt, will prove to be of advantage in detecting small variation in the titre of the neutralising antibody.

Two further points should be stressed in regard to the results of the neutralisation test. It will be seen from table .II.. that except in two cases 20 sera taken before the sixth day after the onset did not protect the mumps infected eggs in a dilution greater than 1:32. This finding suggests, although the number of patients is small, that susceptible persons will not show titres exceeding this level. Later during the infection, from seven days onwards, consistently higher titres are obtained. It would therefore be reasonable to deduce that the presence of the neutralising antibody at higher concentrations in convalescent sera at dilutions of 1:128 or greater, is suggestive of mumps infection. It is also noteworthy that long after the infection the neutralising antibody is present at a low level, but higher than the levels shown by the acute stage sera.

A/

A study of the relationship of the different antibodies to each other has shown that any of the serological tests may be used as a reliable aid in the diagnosis of mumps. As stated in the introductory review, the complement fixation test with 'soluble' antigen has been of great help in the diagnosis of the typical as well as atypical cases of mumps especially during the early stage of the infection. The findings are in agreement with those of Henle and her co-workers who originally pointed the usefulness of the 'soluble' antigen. It is probable that the results of the opposing group of investigators like Aikawa and Meiklejohn (1949), Florman and Kutch (1949), and Feller and Jordan (1950) were at variance because of the different methods employed for preparing the antigens and in performing the complement fixation tests. From the nature of this investigation it is not possible to say whether the above two factors or the use of a different strain of mumps virus by some of these investigators has been responsible for this discrepancy.

It became apparent that in some cases the Anti-S antibody may fail to develop and the titre even in the positive cases is numerically low in comparison/

comparison with those of Anti-V or H.I. and neutralising antibodies. Unless the 'S' antigen is properly standardised many of the positive cases are liable to be diagnosed otherwise, especially when the titre of Anti-S is low. However, it may be asserted that if with in the first week of the disease the Anti-S antibody is present at a significant level even in the absence of other antibodies a presumptive diagnosis of infection with mumps can be made.

All other antibodies besides the Anti-S are, in fact, present at a very low titre and are usually found below the significant level during the acute stage of the disease. Towards the end of the first week a moderate rise of one or the other antibody may be detected in an occasional case. It should be pointed out that the presence of non-specific inhibitors of haemagglutination in the acute stage sera did not interfere with the useful application of the H.I. test even at low dilutions.

Early in the second week of the disease the H.I. and neutralisation tests reveal a moderate rise of the antibodies and the Anti-V is demonstrable at low level. The Anti-S may show a slight rise, but usually reaches a maximum titre at about this time. Towards the end of the second week/

week very high titres of the H.I. and neutralising antibodies are obtained and with properly standardised antigens it is always possible to give a presumptive diagnosis of mumps from the examination of a single serum. The Anti-V also shows a high titre during the third week, particularly towards the end.

It will appear from the results of the H.I. test and the C.F. test with viral antigen that the corresponding antibodies may remain at reasonably high level late after the infection. A similar observation has been made from the examination of normal adult sera (see Section B). Inapparent or subclinical infections may also be responsible for some increase of these antibodies (Henle and Henle, 1949a). For these reasons it is always preferrable to examine two samples of sera and to demonstrate an antibody rise. A high fold-increase in titre is always evident if the initial sample of serum is collected during the first week of the disease.

Thus, whereas the use of soluble antigen has much to commend for an early diagnosis of mumps any of the other tests may be employed for such purpose if two samples of sera are examined. Of these, the H.I. test is simple and reliable and has/

has added advantages over the C.F. test where viral antigen is being used. Firstly, the former detects the antibody earlier than the latter and secondly, the test is possible even with anti-complementary sera. The neutralisation test which is probably more sensitive, is possible with anti-complementary sera, and detects antibodies earlier than the C.F. test with viral antigen or even the H.I. test. The test however has certain disadvantage due to facts that (i) it may not be performed with contaminated serum; (ii) the test serum must be fresh or preserved frozen at a low temperature, and (iii) the technical manipulations require utmost care and experience. The complement fixation test using both the viral and soluble antigens appears to be satisfactory when properly standardised and provided the sera do not show any anti-complementary activity. An additional advantage of this test is that both the antigens keep long at 4°C in contrast to other test antigens.

The results of titrations of the mumps sera against different strains of virus have shown that significant variation in the antibody level of the same serum may occur. It is difficult to believe that/

that these results were due to the variation of response in the host. On the other hand, they are easily explained on the basis of differences among the virus strains and on their relative affinity to react with a particular serum. The precise nature of these variations may be understood only when definite evidence of antigenic dissimilarity of the virus strains is known. In addition, the experimental results emphasise the importance of choosing a suitable strain of the virus for serological titrations. It will also be evident from these results that with the two newly isolated strains of the virus slightly higher values of the antibody levels were obtained. Moreover, one of the strains (Paul strain) also indicated lower titres with acute sera, so that a wider range of sensitivity of the test was obtained. This is of obvious advantage in serological titrations. The evidence also suggests that the discrepancy in the findings of different investigators are at least partly due to the use of different strains of the virus.

SECTION: B

NATURALLY OCCURRING ANTIBODY IN HUMAN SERUM AGAINST THE MUMPS VIRUS

It has been mentioned in the introductory review of the literature that a high proportion of the adult population is relatively resistant to mumps infection. The disease is common in the childhood and early adolescence. It is, therefore, natural to assume that the immunity of the adult population is, mainly, due to the effect of an earlier exposure to the virus. The active immunity may result either from an actual attack of the disease or as a result of subclinical infection, which has been diagnosed lately on serological findings. The wide prevalence of the virus in the nature probably furnishes the necessary antigenic stimuli to maintain this status of immunity.

Although mumps may occur in susceptible persons of any age, it is extremely rare in the neo-natal period. This is probably due to placental transmission of mumps antibodies (Smith, 1945) resembling that of diphtheria and tetanus anti-toxins. It is, therefore, an indirect evidence that immune antibodies are present in the sera of mothers and these are/

are sufficiently effective to give protection to the new born.

In the past, several workers have tried to find out the factor or factors that determine this immunity and to correlate it with the demonstration of antibody by 'skin-tests' and by serological tests. The antibodies to the mumps virus have been detected in sera long after infection (Enders et al.1945a,b; Marris et al.1946; Feller and Jordan, 1950 etc.). In each of these investigations the complement fixation (C.F.) test either alone or along with the H.I. test has been employed for determining the antibody level.

It is now generally agreed that the complement fixing antibody which is detected in majority of the sera, many years after an infection, is probably a true indicator of the immunity status. The neutralising antibody is usually found at a low level in adult sera after the infection (Leymaster and Ward, 1948).

The significance of the level of H.I. antibodies which may persist at a low level long after an infection is open to criticism. This is due to the fact that several non-specific factors might influence the antibody titration and mask the true level of the immune antibody. At least two non-specific factors have been recognised in human and animal sera which inhibit /

inhibit the haemagglutination by viruses.

One which is thermolabile (at 65°C for 30 min.) is possibly associated with γ-globulin (McCrea, 1945); the other is relatively thermostable and is believed to be a 'mucoid' (McCrea, 1948). The latter has been identified as the 'Francis inhibitor' (Francis, 1947) and is now known to be destroyed by substances such as R.D.E. (Cholera filtrate), sodium or potassium periodates and even by the activity of live virus at a temperature of 37°C (Burnet, 1948). Other inhibitors of the haemagglutination such as blood group A and O substances (Burnet et al., 1947) or extracts from red cells (de Burgh et al., 1948; Friedwald et al., 1947) are also mucoid in nature and usually destroyed by the substances described above. Inhibitors have also been detected in the allantoic fluid (Svedmyr, 1948; Hardy and Horsfall, 1948) and they are regarded as of the same type as above (Anderson, 1949). Burnet and Stone (1946) have also found sharp differences in the inhibitory titre of heated serum, containing no influenzal antibody, when red cells from different fowls were used for the H.I. test. They also found that the non-specific inhibitory titre of serum against mumps virus may be exaggerated if group 'O' cells are used for the titration.

While/

While the influence of so many theoretical variables seriously undermine the value of the H.I. test, it is possible that after proper standardisation the test may become useful even for measuring small amount of antibody (Robbins et al. 1949).

Materials and Methods:

Sera: Blood samples were obtained from the following sources:

Group I	Newborn babies (Cord Blood)	14 samples
Group II	Blood-bank donors	36 "
Group III	Adult sera for Wassermann Reaction	10 "
Group IV	Medical Students	54 "
	Total	114 samples

The sera separated from Gr.I, II, and III samples were stored at -36°C and those from Group IV at 4°C after inactivation at 56°C for 30 min.

Virus: Antigens for H.I.&C.F. tests were prepared from Enders Strain. For neutralisation tests the virus pool prepared from the Enders Strain was the same as that described in section A.

Serological Tests: The same methods as described in Chapter II . For the method of treatment of sera with potassium periodate and subsequent titrations for the H.I. antibody, see Chapter III . Any deviation from the/

the routine tests has been mentioned in the experimental procedures.

EXPERIMENTAL AND RESULTS.

EXPERIMENT I :

14 sera from newborn babies were tested for the presence of H.I. antibodies by the usual method of inactivation of the sera at 56°C for 30 min. The sera were also tested unheated and after heating at 66°C for 30 min. The results are shown in table I It was found that unheated serum gave higher titres and there was a tendency of the titre to decrease when the temperature of inactivation is increased. Heating of the sera at 66°C however gave rise to some difficulty in recording the end points. The agglutinated cells settled in atypical forms and the boundary between the inhibition and agglutination was not so clear cut. The titration of the sera after inactivation at 56°C (which is comparable to those of other sera) showed that in about 43% cases there was a titre of 1:128; about 80% of the sera showed a titre of 1:64 or higher.

EXPERIMENT II:

36 sera (from Group II) were titrated for the presence of the H.I. antibody after heat-inactivation (56°C for 30 min.) and after treatment with potassium periodate/

periodate solution. Each serum was tested simultaneously by both these methods. The sera were tested with the same virus and the same batch of red cells. The results are shown in table II . Although the dilutions of the sera were not strictly comparable in the two methods, it will be found that periodate treatment greatly reduces the titre of each serum. Thus, at least 69% of the sera showed a titre of 1:24 and less after the periodate treatment, while the ordinary method of heat inactivation showed a titre of 1:32 in 22% cases.

In spite of the above advantage of periodate treatment, great difficulties were experienced in reading the results. In the lower dilution tubes the pattern of the agglutinated cells were usually atypical. Even in the tubes which showed inhibition, the red cells had a tendency to settle in an irregular broad zone surrounding a central button of cells. The cells were at times discoloured (brownish) in the first two lower dilution tubes and the same effect was also seen in the control tubes (cell + serum without the virus).

EXPERIMENT III:

As in experiment I, the sera from Gr.III normal adults were examined by the H.I. test after inactivation at 56°C and 66°C for 30 minutes. The titrations did not reveal any significant variation in the titres/

titres. Results are shown in the appendix. The results of the titration after inactivation of sera at 56°C for 30 mins. are grouped together with those of Gr.II sera and have analysed towards the end of this section.

EXPERIMENT IV:

15 sera from Gr.II normal adults were tested for the presence of complement fixing antibody using viral antigen only. The results are shown in table IV. and analysed along with the results of other sera in experiment V.

EXPERIMENT V:

Serological titrations to demonstrate the presence of antibody against mumps virus in 54 normal sera and correlation of the findings with the history of previous infection.

Each of the 54 sera (Group IV) was tested for the presence of H.I. and C.F. (both Anti-V and Anti-S) antibodies. A pooled serum prepared from samples which did not contain H.I. and C.F. antibodies was also tested for neutralising capacity. The donors were interrogated for any history of previous infection (clinical) or exposure to mumps patients. The titres of H.I. and C.F. antibodies in individual sera/

sera and the history of infection or exposure are summarised in table IV. The protocol of full titrations are given in appendix D . The results of the antibody titrations will be described at first separately and later the findings from all these experiments with normal sera will be discussed together.

H.I. antibody: The number of sera showing inhibition at a particular titre are shown in table III. It was found that at least 33% (18 out of 54) of the sera showed a titre as high as 1:256. Of these, 4 sera had titres higher than 1:256. 73% of the sera (40 out of 54) had titres of 1:128 or higher and about 96% (52 out of 54) showed titres of 1:64 or higher.

C.F. antibody:

Anti-V: 70% of the sera (38 out of 54) showed a significant (minimum) amount of the antibody and gave fixations at a dilution of 1:4 or higher. The rest of the sera did not contain the anti-V (30%). 50% of the sera (27 out of 54) showed positive fixation at 1:8 or higher and at least in two sera titres as high as 1:64 and 1:32 were observed.

Anti-S: The antibody was detected in a small number of sera. Only in 30% cases (12 out of 54) there was a significant amount of antibody (at 1:4 or higher) present in these sera.

The/

The highest titre seen was 1:16 in one case. The antibody was detected only in those sera which also showed the anti-V antibody.

Neutralising antibody: The pooled serum (from sera Nos. 3, 21, 25, 26, 27 and 34) at 1:16 dilution did not protect any of the 6 eggs inoculated with 100 E.I.D50 of virus. It should be pointed out however that these sera were not preserved frozen and had been inactivated at 56°C for 30 min, before being stored at 4°C.

CORRELATION OF THE HISTORY OF MUMPS AND SEROLOGICAL FINDINGS.

The history of previous infection as obtained from the donors could be summarised as follows:

I) Definite history of infection..... 34 (63%)

II) Definite history of infection + history of contact 38 (70%)

III) Negative history of infection..... 10 (18·5%)

IV) Negative history of infection + history of contact 14 (26%)
(if history of contact are taken as negative history)

V) No history obtained or doubtful history... 6 (11%)

Out of the 34 sera obtained from donors giving definite history of past infection, the C.F. antibody was demonstrated in 27 cases (79·4%). 2 out of 4 donors/

donors who confirmed close contact with mumps patients also showed the C.F. antibody. Thus, if the history of infection and that of contact are considered together, it is possible to show the C.F. antibody in at least 76% of the cases. The C.F. antibody was also detected in 4 out of 10 sera (40%) where a history of mumps was categorically denied. In the series where the history was doubtful or unobtainable, 5 out of 6 sera also contained the C.F. antibody. The presence or absence of the C.F. antibody in all the 54 sera and its correlation with the history of mumps may be shown as follows:

*History +ve		History -ve		Doubtful or no History		Total
Anti-V +ve	Anti-V -ve	Anti-V +ve	Anti-V -ve	Anti-V +ve	Anti-V -ve	
29	9	4	6	5	1	54

* Includes 4 sera with a positive history of 'contact'.

In 33 sera where the C.F. antibody (anti-V) was detected, a positive history of previous infection was obtained in 29 cases (88%). Out of 12 sera which contained the anti-S, a positive history was obtained in 9 cases; in one case there was a negative history and in two others no history could be obtained from the donors. Thus, where anti-S antibody was detected in 9 cases out of 10 (90%) there was also a positive history of past infection.

It/

It has not been possible in every case to observe a correlation between the titres of the anti-V and anti-S antibodies with the time interval since last infection.

In many cases, however, it was found that the sera containing the anti-S had also a comparatively higher titre of the anti-V; a majority of these sera were obtained from donors who suffered from the clinical infection during their early adolescence. The anti-S antibody was detected in only 36% (12 out of 33) of the sera containing the anti-V antibody.

The presence of non-specific factors in serum which inhibit haemagglutination may influence the interpretation of the antibody titres obtained by the H.I. test. However, some of the findings are noteworthy. It was found (see table VII) that of the 34 sera from donors with positive history, a majority (27 sera or 79%) contained the H.I. antibody at a titre of 1:128 or higher.

Except in 2 cases all the 27 sera also contained the anti-V complement fixing antibody. 6 sera with doubtful or unknown history contained H.I. antibody at a titre of 1:128 and over, and these again contained the C.F. antibody in all except one case. Even at a titre of 1:64, 6 out of 12 or 50% of the sera showed the presence of the anti-V antibody at significant levels. It will be observed from the results of the acute stage sera of mumps patients (see section A, this chapter) that the majority of the sera had a titre/

titre of 1:32 only within the first four to five days after the onset of infection. Several months after infection some of the convalescent mumps sera contained the H.I. antibody at titres varying between 1:128 and 1:256 and in each case also contained the anti-V and anti-S antibodies. On this evidence it is possible that the H.I. antibody titres at 1:128 or 1:256 as seen in this experiment was a true measure of the specific antibody.

Antibody levels in adult sera

The combined results of the foregoing experiments (II to V) are given in tables III, IV and V and also shown in the histograms of figure 8.

Of the 100 adult sera titrated by the H.I. test, approximately 70% contained the antibody at a level of1:128 and above. Amongst these about 25% showed a high titre of 1:256.

The anti-V complement fixing antibody was detected at or above the significant level (1:4) in 84% of the sera (44 out of 69). Of the positive sera, at least 45% (31 out of 69) contained the antibody at a level of 1:8 or higher.

In a majority of the sera (70%) the anti-S antibody was negative (less than 1:4). Only in 8% of the positive sera a titre of 1:8 or above could be observed.

DISCUSSION /

TABLE : I

Level of Haemagglutination-Inhibiting Antibody in the Sera (Cord Blood) of 14 Newborn Babies and the Effect of Heat on the Antibody Titre

Serum	Reciprocal of serum dilution(final)			
	32	64	128	256
Unheated	2^^	2	8	2
Heated at 56°C(1/2hr)	3 (21%)ϒ	5 (36%)	6 (43%)	0
Heated at 66°C(1/2hr)	5	6	3	0

^^Number of sera at a particular titre
ϒPercentage of sera at a particular titre

TABLE : II

Comparison of the Effects of a) Heat and b) Periodate on the H.I.titre of 36 Normal Human Sera(Gr.II)

a) Serum heated at 56°C for 30 min.			b)Serum treated with potassium periodate		
Titre (reciprocal)	No. of sera	Percentage (approx.)	Titre (reciprocal)	No. of sera	Percentage (approx.)
32	8	22	24	13	36
64	7	20	24	12	33
128	17	47	48	7	20
256	4	11	96	3	8
			196	1	3

TABLE : III

Level of Haemagglutination-Inhibiting Antibody in 100 Sera from Normal Adults

Serum	Reciprocal of the serum diltn.(final)					Total
	16	32	64	128	256	
Gr.II&III	0*	8	8	23	7	46
Gr.IV	1	1	12	22	18ψ	54
Total	1	9	20	45	25	100

* Number of sera with a particular titre

ψ Includes 4 sera which had the titre higher than 1:256

TABLE : IV

Level of the Complement Fixing Antibody (Anti-V) in 69 Sera from Normal Adults

Serum	Reciprocal of the serum dilution(initial)						Total
	<4	4	8	16	32	64	
Gr.II	9	2	2	2	0	0	15
Gr.IV	16	11	16	9	1	1	54
Total	25	13	18	11	1	1	69
Percentage (approx.)	36	19	26	16	1.5	1.5	-

TABLE :V

Level of the Complement Fixing Antibody(Anti-S) in 54 Sera from Normal Adults

Serum	Reciprocal of the serum dilution(initial)				Total
	<4	4	8	16	
Gr. IV	38	12	3	1	54
Percentage (approx.)	70	22	6	2	-

TABLE : VI

Correlation of History of Mumps Infection and Presence of Antibodies against Mumps and Newcastle Disease Viruses in 54 Students' Sera

Serum No.	Complement fixation titre (Mumps Virus)		Haemagglutination Inhibition titre		HISTORY	AGE WHEN INFECTED (approx.)
	Against Viral Antigen	Against Soluble Antigen	Against Mumps Virus	Against N.D.V.		
1	16	<4	128	16	+	3
2	<4	<4	128	16	=	
3	<4	<4	16	<16	+	8
4	16	4	>256	16	+	14
5	4	<4	256	16	?	
6	16	8	256	64	+	15
7	<4	<4	128	<16	+	7to8
8	8	<4	128	<16	+	7to8
9	32	16	256	64	+	R/1
10	8	4	128	<16	?	
11	4	<4	128	<16	+	4
12	8	8	64	16	+	7to8
13	4	<4	128	32	+	14
14	4	<4	256	<16	=	E/10
15	8	4	256	16	-	
16	8	<4	256	<16	+	13
17	<4	<4	128	<16	-	
18	16	4	128	16	+	4to5
19	4	<4	64	<16	-	
20	16	4	256	16	+	5
21	<4	<4	64	<16	+	6to7
22	4	<4	256	<16	+	3to4
23	8	<4	64	16	-	
24	<4	<4	128	<16	-	
25	<4	<4	64	16	+	10

\+ Positive history of infection
\- Negative history of infection
R/1: history of infection 1 year back
E/10: history of exposure 10 years back
? : No information

TABLE :VI (contd.)

Serum No.	Complement fixation titre (Mumps Virus)		Haemagglutination Inhibition titre		HISTORY (+ve or -ve)	AGE WHEN INFECTED (approx.)
	Against Viral Antigen	Against Soluble Antigen	Against Mumps Virus	Against N.D.V.		
26	<4	<4	64	<16	-	
27	<4	<4	64	<16	-	
28	8	<4	64	<16	C_1	
29	<4	<4	128	<16	C_2	
30	<4	<4	256	16	-	
31	4	<4	128	<16	+	3
32	4	<4	64	<16	+	9
33	8	<4	>256	16	?	
34	<4	<4	32	<16	-	
35	4	<4	256	64	+	9
36	16	4	256	32	+	15
37	8	<4	>256	256	+	R/2
38	<4	<4	128	<16	?	
39	<4	<4	128	<16	+	7to8
40	<4	<4	128	<16	+	4to5
41	4	<4	128	<16	+	3to5
42	8	4	128	32	+	10
43	<4	<4	64	<16	+	7to8
44	16	<4	128	16	+	12
45	8	<4	256	<16	?	?
46	8	<4	256	<16	+	7to8
47	16	4	128	16	D	
48	8	<4	>256	<16	+	16
49	8	<4	128	<16	+	5to6
50	8	<4	128	<16	+	7to8
51	16	<4	256	64	+	10
52	8	<4	64	<16	-	
53	4	<4	128	16	+	10
54	64	4	128	128	+	10

C_1= Close contact with a case
C_2= Close contact 2years ago
R = Recent infection, 2 years before
D = Doubtful history
? :No information

TABLE : VII

Correlation between the history of mumps and the number of sera showing particular titre of H.I. and C.F. antibodies

History of mumps infection	Sera (anti-body)	Titre of sera(reciproc.)					Total
		16	32	64	128	256&>	
Positive	H.I.+ve	1		6	15	12	34
	C.F.+ve			2(1)	13(3)	12(5)	27(9)Ψ
	C.F.-ve	1		4	2		7
Negative (including contacts)	H.I.+ve		1	6	4	3	14
	C.F.+ve			4		2(1)	6(1)
	C.F.-ve		1	2	4	1	8
Doubtful and Unknown	H.I.+ve				3	3	6
	C.F.+ve				2(2)	3	5(2)
	C.F.-ve				1		1

Ψ figure in brackets indicate the number of sera showing anti-S antibody besides the anti-V

DISCUSSION

The results of this investigation confirm the views of epidemiologists that a considerable proportion of the adult population is immune to mumps infection, due to the presence of specific antibodies in their sera. The antibodies have been found to persist at significant levels long after an actual attack of the disease. It cannot, however, be said whether the antibody is 'residual' from the past infection or it has been maintained at a significant level due to repeated exposure to the virus in nature.

There is a good correlation between the serological findings and the positive history of past infection. It could be observed that the H.I. and the C.F. antibodies were maintained in about 79% of cases. The finding is comparable to the results of Maris et al. (1946) who did not find C.F. antibody in about 20% of the sera with positive history of mumps. These workers used the monkey parotid gland antigen for the C.F. test. On the other hand, it was found that a great majority of the sera with antibodies (88%) were obtained from adults giving a positive history.

The slight discrepancy in the correlation of both these findings is possibly due to the following factors:

(1) The antibodies did not persist for long and were/

were therefore below the significant level.

(2) The history was unreliable in certain cases, especially when the infection occurred very early during the childhood.

(3) The antibody formation was due to subclinical infection. The last two possibilities are supported by the indirect finding, that 40% of the sera with a negative history contained specific antibodies. The finding is also in agreement with the figure of 42% reported by Maris and his co-workers.

The correlation is best shown by the sera which contained the anti-S complement fixing antibody. As high as 90% of these sera were obtained from adults with a positive history. However as has been pointed out before, the antibody does not persist long after the infection and was detected only in 35% of the cases with a definite past history of mumps.

It is significant to note that the anti-S antibody is absent from a majority (70%) of sera selected at random. Even in positive sera the level of the antibody is very low. These facts emphasise that a suspected infection of mumps in adults may be diagnosed if a high level of the anti-S is present. Since the anti-V or the H.I. antibodies may be defected at a high level in a majority of the adult sera, their values under such conditions will be limited; they are useful only when a four-fold rise in titre can be demonstrated.

The/

The close correlation of the positive serological findings with the cases of past infection makes it possible to identify the person who due to the presence of specific antibodies may be expected to be relatively immune. It cannot however be decided from the nature of the experiments whether the presence of the H.I. or of the C.F. antibody determined the immunity.

It was shown in the previous section that a majority of the acute stage sera from mumps, especially during the initial 3-4 days of the disease, contained the H.I. antibody at a level of 1:32. In contrast, about 80% of the newborn babies have been found to possess the antibody at 1:64 or above and at least 43% of them had a titre of 1:128. In presence of a high level of the H.I. antibody in adult sera it is probable that the immunity during the neo-natal period is due to actual transmission of the maternal antibody to the infant. It confirms an earlier report by Maris et al. (1946) concerning the transmission of mumps complement fixing antibodies to the infants.

The above results also indicate that at some period during the infancy there is a gradual waning of the antibody to levels of 1:32 or lower and at this stage the infants become susceptible to the infection.

In the past, considerable controversy has been raised on the value of the H.I. technique for titration of the specific antibody in mumps. It has been shown/

shown in Section A that the methods described by Robbins et al. (1949), which was also used in the present study, proved as reliable as the C.F. test for the detection of increases in titre in paired mumps sera. Similar findings have been reported by Feller and Jordan (1950) who used the same technique.

The results of the H.I. test with normal adult sera have shown that there is a similar co-relation with the complement fixation test (viral antigen). In a majority of sera the level of the H.I. antibody was also found to vary with the positive or the negative history of previous infection. Thus, only in two cases the antibody was at a relatively low titre when there was a positive history (34 cases). Of the sera with a negative history the H.I. antibody was detected at a high level in 4 cases and in three of these the C.F. antibody was also present. It is probable that these sera were obtained from the inapparent or subclinical infections as discussed before.